(54) Title: HIGH AFFINITY TGFγ NUCLEIC ACID LIGANDS AND INHIBITORS

(57) Abstract: Methods are described for the identification and preparation of high-affinity nucleic acid ligands to TGFβ2. Included in the invention are specific RNA ligands to TGFβ2 identified by the SELEX method. Also included are RNA ligands that inhibit the interaction of TGFβ2 with its receptor.

HIGH AFFINITY TGFß NUCLEIC ACID LIGANDS AND INHIBITORS

FIELD OF THE INVENTION

Described herein are methods for identifying and preparing high affinity nucleic

5   acid ligands that bind human transforming growth factor β2 (TGFβ2). The method

utilized herein for identifying such nucleic acid ligands is called SELEX, an acronym for

Systematic Evolution of Ligands by EXponential Enrichment. This invention includes

high affinity nucleic acids of human TGFβ2. Further disclosed are RNA ligands to

TGFβ2. Also included are oligonucleotides containing nucleotide derivatives modified at

10   the 2' position of the pyrimidines. Additionally disclosed are ligands to TGFβ2 containing

2'-OCH$_3$ purine modifications that may have higher stability in serum and in animals. This

invention also includes high affinity nucleic acid inhibitors of TGFβ2. The

oligonucleotide ligands of the present invention are useful in any process in which binding

to TGFβ2 is required. This includes, but is not limited to, their use as pharmaceuticals,

15   diagnostics, imaging agents, and immunohistochemical reagents.

BACKGROUND OF THE INVENTION

Transforming growth factor betas (TGFβs) are part of a superfamily of proteins

that includes inhibins, activins, bone morphogenetic and osteogenic proteins,

20   growth/differentiation factors, Mullerian-inhibiting substance, decapentaplegic and 60A

(*Drosophila*), daf-7 and unc-129 (*C. elegans*), and vg1 (*Xenopus*) (Schlunegger and

Grutter (1992) Nature 358:430-434). Three TGFβ isotypes, TGFβ1, TGFβ2, and TGFβ3,

exist in mammals. There is about 80% sequence identity between any pair of mammalian

TGFβs. TGFβs bind to at least 5 receptors, but only 2 or 3 of them (types I, II and

25   possibly V) are signaling receptors. The intracellular signaling pathways activated by

TGFβs involve SMAD proteins and are being intensively studied (Padgett *et al.* (1998)

Pharmacol Ther 78:47-52). The signaling receptors are found on a variety of cells. In

turn, a variety of cells express TGFβs.

TGFβs are synthesized as precursors composed of latency-associated protein

30   (LAP) at the amino terminus and mature TGFβ at the carboxyl terminus. The precursor is

cleaved and assembles as a homodimer. TGFβs are secreted from cells bound to LAP and

latent TGFβ binding proteins (LTBPs). Latent TGFβs are released from LAP and LTBP

2

and become active by a relatively uncharacterized mechanism that may involve proteolysis by plasmin or regulation by thrombospondin (Crawford *et al.* (1998) Cell 93:1159-70). The mature, released TGFβ homodimer has a combined molecular weight of ~25000 daltons (112 amino acids per monomer). TGFβ1 and TGFβ2 bind heparin and there are

5   indications that basic amino acids at position 26 are required for heparin binding (Lyon *et al.* (1997) Jour. Biol. Chem. 272:18000-18006).

The structure of TGFβ2 has been determined using x-ray crystallography (Daopin *et al.* (1992) Science 257:369-373; Schlunegger and Grutter (1992) Nature 358:430-434) and is very similar to the structure of TGFβ1. TGFβs belong to a structural family of

10   proteins called the "cysteine knot" proteins that includes vascular endothelial growth factor, nerve growth factor, human chorionic gonadotropin, and platelet-derived growth factor. These proteins are structurally homologous, but have only 10-25% primary sequence homology.

The biological activities of the TGFβs vary (Moses (1990) Growth Factors from

15   Genes to Clinical Application 141-155; Wahl (1994) J. Exp. Med. 180:1587-1590). In some cases they inhibit cell proliferation (Robinson *et al.* (1991) Cancer Res. 113:6269-6274) and in other cases they stimulate it (Fynan and Reiss (1993) Crit. Rev. Oncogenesis 4:493-540). They regulate extracellular matrix formation and remodeling (Koli and Arteaga (1996) Jour. Mammary Gland Biol. and Neoplasia 1:373-380). TGFβs are also

20   are very potent immunosuppressants (Letterio and Roberts (1998) Ann. Rev. Immunol. 16:137-161). TGFβs are thought to play a significant role in fibrotic diseases, preventing the immune system from rejecting tumors (Fakhrai *et al.* (1996) Proc. Natl. Acad. USA 93:2090-2914), cancer cell growth (Koli and Arteaga (1996) J. Mammary Gland Bio. and Neoplasia 1:373-380; Reiss and Barcellos-Hoff (1997) Breast Cancer Res. and Treatment

25   45:81-85; Jennings and Pietenpol (1998) J. Neurooncol. 36:123-140), and tumor metastasis. They may have ancillary roles in autoimmune and infectious diseases. Inhibition of TGFβ2 by an expressed antisense RNA (Fakhrai *et al.* (1996) Proc. Natl. Acad. USA 93:2090-2914) and by exogenous antisense oligonucleotides (Marzo *et al.* (1997) Cancer Research 57:3200-3207) has been reported to prevent glioma formation in

30   rats.

3

The gene for mouse TGFβ2 has been deleted (Sanford *et al.* (1997) Development 124: 2659-2670). Mice lacking TGFβ2 function die near birth and have aberrant epithelial-mesencymal interactions that lead to developmental defects in the heart, eye, ear, lung, limb, craniofacial area, spinal cord, and urogenital tracts. These defects, for the most

5   part, do not overlap abnormalities that have been observed in TGFβ1 and TGFβ3 knockout mice. TGFβs have also been overexpressed in cell lines or transgeneic mice (Koli and Arteaga (1996) J. Mammary Gland Bio. and Neoplasia 1:373-380; Bottinger *et al.* (1997) Kidney Int. 51:1355-1360; Bottinger and Kopp (1998) Miner Electrolyte Metab 24:154-160) with a variety of effects.

10   A method for the *in vitro* evolution of nucleic acid molecules with high affinity binding to target molecules has been developed. This method, Systematic Evolution of Ligands by EXponential enrichment, termed SELEX, is described in U.S. Patent Application Serial No. 07/536,428, filed June 11, 1990, entitled "Systematic Evolution of Ligands by Exponential Enrichment," now abandoned, U.S. Patent No. 5,475,096, entitled

15   "Nucleic Acid Ligands," and U.S. Patent No. 5,270,163, entitled "Methods for Identifying Nucleic Acid Ligands" (see also WO91/19813), each of which is specifically incorporated herein by reference in its entirety. Each of these applications, collectively referred to herein as the SELEX Patent Applications, describe a fundamentally novel method for making a nucleic acid ligand to any desired target molecule.

20   The SELEX method involves selection from a mixture of candidate oligonucleotides and step-wise iterations of binding, partitioning and amplification, using the same general selection theme, to achieve virtually any desired criterion of binding affinity and selectivity. Starting from a mixture of nucleic acids, preferably comprising a segment of randomized sequence, the SELEX method includes steps of contacting the

25   mixture with the target under conditions favorable for binding, partitioning unbound nucleic acids from those nucleic acids which have bound to target molecules, dissociating the nucleic acid-target complexes, amplifying the nucleic acids dissociated from the nucleic acid-target complexes to yield a ligand-enriched mixture of nucleic acids, then reiterating the steps of binding, partitioning, dissociating and amplifying through as many

30   cycles as desired to yield high affinity nucleic acid ligands to the target molecule.

The basic SELEX method may be modified to achieve specific objectives. For

4

example, U.S. Patent Application Serial No. 07/960,093, filed October 14, 1992, entitled "Method for Selecting Nucleic Acids on the Basis of Structure," now abandoned, describes the use of SELEX in conjunction with gel electrophoresis to select nucleic acid molecules with specific structural characteristics, such as bent DNA (see U.S. Patent No. 5,707,796).

5   U.S. Patent Application Serial No. 08/123,935, filed September 17, 1993, entitled "Photoselection of Nucleic Acid Ligands," now abandoned, describes a SELEX based method for selecting nucleic acid ligands containing photoreactive groups capable of binding and/or photocrosslinking to and/or photoinactivating a target molecule. U.S. Patent Application Serial No. 08/134,028, filed October 7, 1993, entitled "High-Affinity

10   Nucleic Acid Ligands That Discriminate Between Theophylline and Caffeine," now abandoned, describes a method for identifying highly specific nucleic acid ligands able to discriminate between closely related molecules, termed "Counter-SELEX" (see U.S. Patent No. 5,580,737). U.S. Patent Application Serial No. 08/143,564, filed October 25, 1993, entitled "Systematic Evolution of Ligands by EXponential Enrichment: Solution

15   SELEX," now abandoned, (see also U.S. Patent No. 5,567,588) and U.S. Patent No. 5,861,254, entitled "Flow Cell SELEX," describe SELEX-based methods which achieve highly efficient partitioning between oligonucleotides having high and low affinity for a target molecule. U.S. Patent No 5,496,938, entitled " Nucleic Acid Ligands to HIV-RT and HIV-1 Rev," describes methods for obtaining improved nucleic acid ligands after the

20   SELEX process has been performed. U.S. Patent No. 5,705,337, entitled "Systematic Evolution of Ligands by EXponential Enrichment: Chemi-SELEX," describes methods for covalently linking a ligand to its target.

          The SELEX method encompasses the identification of high-affinity nucleic acid ligands containing modified nucleotides conferring improved characteristics on the ligand,

25   such as improved *in vivo* stability or delivery. Examples of such modifications include chemical substitutions at the ribose and/or phosphate and/or base positions. Specific SELEX-identified nucleic acid ligands containing modified nucleotides are described in U.S. Patent Application Serial No. 08/117,991, filed September 8, 1993, entitled "High Affinity Nucleic Acid Ligands Containing Modified Nucleotides," now abandoned, that

30   describes oligonucleotides containing nucleotide derivatives chemically modified at the 5- and 2'-positions of pyrimidines, as well as specific RNA ligands to thrombin containing 2'-

amino modifications (see U.S. Patent No. 5,660,985). U.S. Patent Application Serial No. 08/134,028, *supra*, describes highly specific nucleic acid ligands containing one or more nucleotides modified with 2'-amino (2'-NH$_2$), 2'-fluoro (2'-F), and/or 2'-O-methyl (2'-OMe).). U.S. Patent Application Serial No. 08/264,029, filed June 22, 1994, entitled

5   "Novel Method of Preparation of Known and Novel 2' Modified Nucleosides by Intramolecular Nucleophilic Displacement," now abandoned, describes oligonucleotides containing various 2'-modified pyrimidines. PCT/US98/00589 (WO 98/18480), filed January 7, 1998, entitled "Bioconjugation of Oligonucleotides," describes a method for identifying bioconjugates to a target comprising nucleic acid ligands derivatized with a

10   molecular entity exclusively at the 5'-position of the nucleic acid ligands.

The SELEX method encompasses combining selected oligonucleotides with other selected oligonucleotides and non-oligonucleotide functional units as described in U.S. Patent No. 5,637,459, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Chimeric SELEX," and U.S. Patent No. 5,683,867, entitled "Systematic

15   Evolution of Ligands by Exponential Enrichment: Blended SELEX," respectively. These applications allow the combination of the broad array of shapes and other properties, and the efficient amplification and replication properties, of oligonucleotides with the desirable properties of other molecules. The full text of the above described patent applications, including but not limited to, all definitions and descriptions of the SELEX process, are

20   specifically incorporated herein by reference in their entirety.

BRIEF SUMMARY OF THE INVENTION

The present invention includes methods of identifying and producing nucleic acid ligands to transforming growth factor beta (TGFβ2) and the nucleic acid ligands so

25   identified and produced. In particular, RNA sequences are provided that are capable of binding specifically to TGFβ2. Also included are oligonucleotides containing nucleotide derivatives modified at the 2' position of the pyrimidines. Specifically included in the invention are the RNA ligand sequences shown in **Tables 5, 7, 8, 11, 13, 14, 16-19** and **Figure 9** (SEQ ID NOS:21-108 and 128-193). Also included in this invention are RNA

30   ligands of TGFβ2 that inhibit the function of TGFβ2. Also described herein are 2'OMe-modified nucleic acid ligands of TGFβ1.

6

Further included in this invention is a method of identifying nucleic acid ligands and nucleic acid ligand sequences to TGFβ2, comprising the steps of (a) preparing a candidate mixture of nucleic acids, (b) contacting the candidate mixture of nucleic acids with TGFβ2, (c) partitioning between members of said candidate mixture on the basis of

5   affinity to TGFβ2, and (d) amplifying the selected molecules to yield a mixture of nucleic acids enriched for nucleic acid sequences with a relatively higher affinity for binding to TGFβ2.

More specifically, the present invention includes the RNA ligands to TGFβ2, identified according to the above-described method, including those ligands shown in

10   Tables 5, 7, 8, 11, 13, 14, 16-19 and Figure 9 (SEQ ID NOS:21-108 and 128-193). Also included are nucleic acid ligands to TGFβ2 that are substantially homologous to any of the given ligands and that have substantially the same ability to bind TGFβ2 and inhibit the function of TGFβ2. Further included in this invention are nucleic acid ligands to TGFβ2 that have substantially the same structural form as the ligands presented herein and that

15   have substantially the same ability to bind TGFβ2 and inhibit the function of TGFβ2.

The present invention also includes other modified nucleotide sequences based on the nucleic acid ligands identified herein and mixtures of the same.

BRIEF DESCRIPTION OF THE FIGURES

20   Figure 1 shows a flow chart summarizing the various SELEX experiments done with TGFβ2. The length of the arrowheads corresponds to the round number shown to the left. Connected arrowheads indicate branches in the SELEX experiments where a pool was used to start a new branch. Under each arrowhead the fold improvement in affinity is also shown.

25   Figure 2 shows activity of TGFβ2 following amine coupling on a BIAcore carboxymethylcellulose (CM5) chip. A CM5 chip was loaded with TGFβ2 using NHS-EDC coupling as described in the Example 1 at about 18, 718, and 1692 response units for flow cell (FC) 1, 2 and 3, respectively. FC-4 was left blank as a control and was used to normalize the signals from the other FCs. The chip was then exposed to 10 nM of either

30   latency associated peptide (LAP) (Figure 2A) or TGFβ soluble receptor III (sRIII) (Figure

7

2B) at 20 µL/min in binding buffer. Data were collected for an association and a

dissociation phase as shown. The signal from FC-4 was subtracted from the other FCs.

.Figure 3 shows affinity improvement during the spr SELEX. A CM5 chip was

loaded with TGFβ2 using NHS-EDC coupling as described in Example 1 at about 18, 718,

5   and 1692 response units for flow cell (FC) 1, 2, and 3, respectively. FC-4 was left blank

as a control and was used to normalize the signals from the other FCs. The chip was then

exposed to 1 µM of RNA pools from the SELEX rounds (Rd) as shown at 20 µL/min in

binding buffer. Data were collected for an association and a dissociation phase as shown.

The signal from FC-4 was subtracted from the other FCs.

10   Figure 4 shows nitrocellulose filter binding curves with pools from the spr

SELEX. High specific activity internally labeled RNA was used from rounds (R) as

shown. Labeled RNA was incubated with various concentrations of TGFβ2 in the

presence of ~100,000 fold molar excess unlabeled tRNA. Bound RNA was partitioned by

nitrocellulose filtration and quantitated. Data were analyzed as described in Example 1.

15   Figure 5 shows nitrocellulose filter binding curves with various pools. High

specific activity internally labeled RNA was used from rounds (R) as shown. Labeled

RNA was incubated with various concentrations of TGFβ2 (no competitor tRNA was

used). Bound RNA was partitioned by nitrocellulose filtration and quantitated. Data were

analyzed as described in Example 1.

20   Figure 6 shows specificity of the bioactivity of lead TGFβ1 and TGFβ2 aptamers

and comparison with commercial antibody preparations. RNA was either synthesized by

phosphoramidite chemistry (NX22283) (SEQ ID NO:114) or by in vitro transcription.

Indicator cells (mink lung epithelial cells) were incubated with either TGFβ1, TGFβ2 or

TGFβ3 and dilutions of RNA or antibody as described. The extent of cell proliferation

25   was measured by ^{3}H-thymidine incorporation and the data were analyzed as described.

The points represent an average of n=2-6 and error bars are standard errors. Symbols

designated by TGFβ1, TGFβ2 or TGFβ3 indicate data obtained from cells treated with

either TGFβ1, TGFβ2 or TGFβ3, respectively. MAB and pAB designate monoclonal and

polyclonal antibodies, respectively. Random, NX22283, and 40-03 designate the use of

30   random RNA, the TGFβ2, or the TGFβ1 lead aptamer, respectively. The aptamer 40-03

8

was described in the U.S. Patent Application Serial No. 09/046,247, filed March 23, 1998, entitled "High-Affinity High Affinity TGFβ Nucleic Acid Ligands and Inhibitors."

Figure 7 shows boundaries of TGFβ2 ligands 14-1 (SEQ ID NO:72), 21-21 (SEQ ID NO:87), and 21-4 (SEQ ID NO:86). RNA aptamers were end labeled at the 5' end

5   (3'B) or at the 3' end (5'B), partially hydrolyzed at high pH, and partitioned for binding to TGFβ2 by nitrocellulose filtration as described in the Example 1. The amounts of TGFβ2 used for binding partitioning is as shown. Recovered RNA was analyzed on high resolution sequencing gels and visualized by autoradiography. Unselected hydrolyzed RNA was used as a marker (Alk. hydr.) to align the banding pattern to the sequence of

10 each ligand. The observed boundary bands are shown with (*) and their position in the sequence pattern is shown by arrowheads. No protein and input lanes show the background binding to nitrocellulose and the starting unhydrolyzed RNA. The observed boundaries for each ligand is summarized at the bottom of the figure.

Figure 8 shows the putative structures of TGFβ2 aptamers. The minimal required

15 sequences were fit into similar structures. Ligand 14i-1t5-41 (SEQ ID NO:131) and 21a-4(ML-110) (SEQ ID NO:144) were transcribed in vitro and contained extra bases at their 5' ends (shown in lower case) to allow efficient in vitro transcription. Bold-faced letters indicate positions that are identical to invariant positions of the biased SELEX with the 21-21 sequence (SEQ ID NO:93).

20 Figure 9 shows the molecular structure of NX22323 40k PEG (SEQ ID NO:115). rG = 2'OH G; rA = 2'-OH A; fU = 2'FU; fC = 2'FC.

Figure 10 shows the putative structure of lead truncate ligand CD70 (SEQ ID NO:216). Lower case letters indicate positions requiring 2'OH and ● indicates GU base pairing.

25 Figure 11 shows the pharmacokinetics of TGFβ aptamer in sprague dawley rats (dose 1 mg/kg).

Figure 12 shows the results of three separate PAI-Luciferase assays performed on MLEC with TGFβ1 and TGFβ1 aptamer M22 (SEQ ID NO:215). The negative control is an unrelated aptamer. The TGFβ concentration was held constant at 20 pM for 16 hours.

30 Figure 13 shows the results of three separate PAI-Luciferase assays performed on MLEC with TGFβ2 and the TGFβ2 aptamer NX22421 (SEQ ID NO:186). The negative

9

control is an unrelated aptamer. The TGFβ concentration was held constant at 10 pM for
16 hours.

DETAILED DESCRIPTION OF THE INVENTION

5   This application describes high-affinity nucleic acid ligands to TGFβ2 identified
through the method known as SELEX. SELEX is described in U.S. Patent Application
Serial No. 07/536,428, entitled "Systematic Evolution of Ligands by EXponential
Enrichment," now abandoned, U.S. Patent No. 5,475,096, entitled "Nucleic Acid
Ligands," and U.S. Patent No. 5,270,163, entitled "Methods for Identifying Nucleic Acid
10   Ligands,"(see also WO91/19813). These applications, each specifically incorporated
herein by reference in its entirety, are collectively called the SELEX Patent Applications.
Nucleic acid ligands to TGFβ have been identified through the SELEX method. These
TGFβ nucleic acid ligands are described in U. S. Patent No. 5,731,144, U.S. Patent
Application Serial No. 09/046,247, filed March 23, 1998, both entitled, "High Affinity
15   TGFβ Nucleic Acid Ligands and Inhibitors," and U.S. Patent Application Serial No.
09/275,850, filed March 24, 1999, entitled "Truncation SELEX Method." These
applications are specifically incorporated herein by reference in their entirety.
          Certain terms used to described the invention herein are defined as follows.
          "Nucleic Acid Ligand" as used herein is a non-naturally occurring nucleic acid
20   having a desirable action on a target. Nucleic acid ligands are also referred to herein as
"aptamers." A desirable action includes, but is not limited to, binding of the target,
catalytically changing the target, reacting with the target in a way which modifies/alters
the target or the functional activity of the target, covalently attaching to the target as in a
suicide inhibitor, and facilitating the reaction between the target and another molecule. In
25   a preferred embodiment, the desirable action is specific binding to a target molecule, such
target molecule being a three dimensional chemical structure other than a polynucleotide
that binds to the nucleic acid ligand through a mechanism which predominantly depends
on Watson/Crick base pairing or triple helix binding, wherein the nucleic acid ligand is not
a nucleic acid having the known physiological function of being bound by the target
30   molecule. Nucleic acid ligands include nucleic acids that are identified from a candidate
mixture of nucleic acids, said nucleic acid ligand being a ligand of a given target by the

method comprising: a) contacting the candidate mixture with the target, wherein nucleic acids having an increased affinity to the target relative to the candidate mixture may be partitioned from the remainder of the candidate mixture; b) partitioning the increased affinity nucleic acids from the remainder of the candidate mixture; and c) amplifying the

5   increased affinity nucleic acids to yield a ligand-enriched mixture of nucleic acids.

"Candidate Mixture" is a mixture of nucleic acids of differing sequence from which to select a desired ligand. The source of a candidate mixture can be from naturally-occurring nucleic acids or fragments thereof, chemically synthesized nucleic acids, enzymatically synthesized nucleic acids or nucleic acids made by a combination of the

10   foregoing techniques. In a preferred embodiment, each nucleic acid has fixed sequences surrounding a randomized region to facilitate the amplification process.

"Nucleic Acid" means either DNA, RNA, single-stranded or double-stranded and any chemical modifications thereof. Modifications include, but are not limited to, those which provide other chemical groups that incorporate additional charge, polarizability,

15   hydrogen bonding, electrostatic interaction, and fluxionality to the nucleic acid ligand bases or to the nucleic acid ligand as a whole. Such modifications include, but are not limited to, 2'-position sugar modifications, 5-position pyrimidine modifications, 8-position purine modifications, modifications at exocyclic amines, substitution of 4-thiouridine, substitution of 5-bromo or 5-iodo-uracil, backbone modifications, methylations, unusual

20   base-pairing combinations such as the isobases isocytidine and isoguanidine and the like. Modifications can also include 3' and 5' modifications such as capping.

"SELEX" methodology involves the combination of selection of nucleic acid ligands which interact with a target in a desirable manner, for example binding to a protein, with amplification of those selected nucleic acids. Iterative cycling of the

25   selection/amplification steps allows selection of one or a small number of nucleic acids which interact most strongly with the target from a pool which contains a very large number of nucleic acids. Cycling of the selection/amplification procedure is continued until a selected goal is achieved. In the present invention, the SELEX methodology is employed to obtain nucleic acid ligands to TGFβ2. The SELEX methodology is described

30   in the SELEX Patent Applications.

"**Target**" means any compound or molecule of interest for which a ligand is desired. A target can be a protein, peptide, carbohydrate, polysaccharide, glycoprotein, hormone, receptor, antigen, antibody, virus, substrate, metabolite, transition state analog, cofactor, inhibitor, drug, dye, nutrient, growth factor, etc. without limitation. In this

5   application, the target is TGFβ2.

In its most basic form, the SELEX process may be defined by the following series of steps:

1) A candidate mixture of nucleic acids of differing sequence is prepared. The candidate mixture generally includes regions of fixed sequences (i.e., each of the members

10   of the candidate mixture contains the same sequences in the same location) and regions of randomized sequences. The fixed sequence regions are selected either: a) to assist in the amplification steps described below; b) to mimic a sequence known to bind to the target; or c) to enhance the concentration of a given structural arrangement of the nucleic acids in the candidate mixture. The randomized sequences can be totally randomized (i.e., the

15   probability of finding a base at any position being one in four) or only partially randomized (e.g., the probability of finding a base at any location can be selected at any level between 0 and 100 percent).

2) The candidate mixture is contacted with the selected target under conditions favorable for binding between the target and members of the candidate mixture. Under

20   these circumstances, the interaction between the target and the nucleic acids of the candidate mixture can be considered as forming nucleic acid-target pairs between the target and those nucleic acids having the strongest affinity for the target.

3) The nucleic acids with the highest affinity for the target are partitioned from those nucleic acids with lesser affinity to the target. Because only an extremely small

25   number of sequences (and possibly only one molecule of nucleic acid) corresponding to the highest affinity nucleic acids exist in the candidate mixture, it is generally desirable to set the partitioning criteria so that a significant amount of the nucleic acids in the candidate mixture (approximately 5-50%) are retained during partitioning.

4) Those nucleic acids selected during partitioning as having the relatively higher

30   affinity to the target are then amplified to create a new candidate mixture that is enriched in nucleic acids having a relatively higher affinity for the target.

12

5) By repeating the partitioning and amplifying steps above, the newly formed candidate mixture contains fewer and fewer weakly binding sequences, and the average degree of affinity of the nucleic acids to the target will generally increase. Taken to its extreme, the SELEX process will yield a candidate mixture containing one or a small

5   number of unique nucleic acids representing those nucleic acids from the original candidate mixture having the highest affinity to the target molecule.

The SELEX Patent Applications describe and elaborate on this process in great detail. Included are targets that can be used in the process, methods for partitioning nucleic acids within a candidate mixture, and methods for amplifying partitioned nucleic

10   acids to generate enriched candidate mixture. The SELEX Patent Applications also describe ligands obtained to a number of target species, including both protein targets where the protein is and is not a nucleic acid binding protein.

The SELEX method further encompasses combining selected nucleic acid ligands with lipophilic or non-immunogenic, high molecular weight compounds in a diagnostic or

15   therapeutic complex as described in U.S. Patent No. 6,011,020, "Nucleic Acid Ligand Complexes." VEGF nucleic acid ligands that are associated with a lipophilic compound, such as diacyl glycerol or dialkyl glycerol, in a diagnostic or therapeutic complex are described in U.S. Patent No. 5,859,228, entitled "Vascular Endothelial Growth Factor (VEGF) Nucleic Acid Ligand Complexes." VEGF nucleic acid ligands that are associated

20   with a lipophilic compound, such as a glycerol lipid, or a non-immunogenic, high molecular weight compound, such as polyalkylene glycol, are further described in U.S. Patent Application Serial No. 08/897,351, filed July 21, 1997, entitled "Vascular Endothelial Growth Factor (VEGF) Nucleic Acid Ligand Complexes". VEGF nucleic acid ligands that are associated with a non-immunogenic, high molecular weight compound or lipophilic

25   compound are also further described in PCT/US 97/18944 (WO 98/18480), filed October 17, 1997, entitled "Vascular Endothelial Growth Factor (VEGF) Nucleic Acid Ligand Complexes." Each of the above described patent applications which describe modifications of the basic SELEX procedure are specifically incorporated by reference herein in their entirety.

30   In certain embodiments of the present invention it is desirable to provide a complex comprising one or more nucleic acid ligands to TGFβ2 covalently linked with a non-

13

immunogenic, high molecular weight compound or lipophilic compound. A complex as used herein describes the molecular entity formed by the covalent linking of the nucleic acid ligand of TGFβ2 to a non-immunogenic, high molecular weight compound. A non-immunogenic, high molecular weight compound is a compound between approximately 100

5   Da to 1,000,000 Da, more preferably approximately 1000 Da to 500,000 Da, and most preferably approximately 1000 Da to 200,000 Da, that typically does not generate an immunogenic response. For the purposes of this invention, an immunogenic response is one that causes the organism to make antibody proteins. In one preferred embodiment of the invention, the non-immunogenic, high molecular weight compound is a polyalkylene glycol.

10   In the most preferred embodiment, the polyalkylene glycol is polyethylene glycol (PEG). More preferably, the PEG has a molecular weight of about 10-80K. Most preferably, the PEG has a molecular weight of about 20-45K. In certain embodiments of the invention, the non-immunogenic, high molecular weight compound can also be a nucleic acid ligand.

       In another embodiment of the invention it is desirable to have a complex comprised

15   of a nucleic acid ligand to TGFβ2 and a lipophilic compound. Lipophilic compounds are compounds that have the propensity to associate with or partition into lipid and/or other materials or phases with low dielectric constants, including structures that are comprised substantially of lipophilic components. Lipophilic compounds include lipids as well as non-lipid containing compounds that have the propensity to associate with lipid (and/or other

20   materials or phases with low dielectric constants). Cholesterol, phospholipid, and glycerol lipids, such as dialkylglycerol, diacylglycerol, and glycerol amide lipids are further examples of lipophilic compounds. In a preferred embodiment, the lipophilic compound is a glycerol lipid.

       The non-immunogenic, high molecular weight compound or lipophilic compound

25   may be covalently bound to a variety of positions on the nucleic acid ligand to TGFβ2, such as to an exocyclic amino group on the base, the 5-position of a pyrimidine nucleotide, the 8-position of a purine nucleotide, the hydroxyl group of the phosphate, or a hydroxyl group or other group at the 5' or 3' terminus of the nucleic acid ligand to TGFβ2. In embodiments where the lipophilic compound is a glycerol lipid, or the non-immunogenic, high molecular

30   weight compound is polyalkylene glycol or polyethylene glycol, preferably the non-immunogenic, high molecular weight compound is bonded to the 5' or 3' hydroxyl of the

14

phosphate group thereof. In the most preferred embodiment, the lipophilic compound or non-immunogenic, high molecular weight compound is bonded to the 5' hydroxyl of the phosphate group of the nucleic acid ligand. Attachment of the non-immunogenic, high molecular weight compound or lipophilic compound to the nucleic acid ligand of TGFβ can

5   be done directly or with the utilization of linkers or spacers.

A "linker" is a molecular entity that connects two or more molecular entities through covalent bonds or non-covalent interactions, and can allow spatial separation of the molecular entities in a manner that preserves the functional properties of one or more of the molecular entities. A linker can also be known as a spacer.

10   The complex comprising a nucleic acid ligand to TGFβ2 and a non-immunogenic, high molecular weight compound or lipophilic compound can be further associated with a lipid construct. Lipid constructs are structures containing lipids, phospholipids, or derivatives thereof comprising a variety of different structural arrangements which lipids are known to adopt in aqueous suspension. These structures include, but are not limited to, lipid

15   bilayer vesicles, micelles, liposomes, emulsions, lipid ribbons or sheets, and may be complexed with a variety of drugs and components which are known to be pharmaceutically acceptable. In the preferred embodiment, the lipid construct is a liposome. The preferred liposome is unilamellar and has a relative size less than 200 nm. Common additional components in lipid constructs include cholesterol and alpha-tocopherol, among others. The

20   lipid constructs may be used alone or in any combination which one skilled in the art would appreciate to provide the characteristics desired for a particular application. In addition, the technical aspects of lipid constructs and liposome formation are well known in the art and any of the methods commonly practiced in the field may be used for the present invention.

The SELEX method further comprises identifying bioconjugates to a target.

25   Copending PCT Patent Application No. US98/00589 (WO 98/30720), filed January 7, 1998, entitled "Bioconjugation of Oligonucleotides," describes a method for enzymatically synthesizing bioconjugates comprising RNA derivatized exclusively at the 5'-position with a molecular entity, and a method for identifying bioconjugates to a target comprising nucleic acid ligands derivatized with a molecular entity exclusively at the 5'-position of the

30   nucleic acid ligands. A "bioconjugate" as used herein refers to any oligonucleotide which has been derivatized with another molecular entity. In the preferred embodiment, the

molecular entity is a macromolecule. As used herein, a "macromolecule" refers to a large organic molecule. Examples of macromolecules include, but are not limited to nucleic acids, oligonucleotides, proteins, peptides, carbohydrates, polysaccharides, glycoproteins, lipophilic compounds, such as cholesterol, phospholipids, diacyl glycerols and dialkyl glycerols, hormones, drugs, non-immunogenic high molecular weight compounds, fluorescent, chemiluminescent and bioluminescent marker compounds, antibodies and biotin, etc. without limitation. In certain embodiments, the molecular entity may provide certain desirable characteristics to the nucleic acid ligand, such as increasing RNA hydrophobicity and enhancing binding, membrane partitioning and/or permeability. Additionally, reporter molecules, such as biotin, fluorescein or peptidyl metal chelates for incorporation of diagnostic radionuclides may be added, thus providing a bioconjugate which may be used as a diagnostic agent.

The methods described herein and the nucleic acid ligands identified by such methods are useful for both therapeutic and diagnostic purposes. Therapeutic uses include the treatment or prevention of diseases or medical conditions in human patients. Therapeutic uses may also include veterinary applications.

Diagnostic utilization may include both *in vivo* or *in vitro* diagnostic applications. The SELEX method generally, and the specific adaptations of the SELEX method taught and claimed herein specifically, are particularly suited for diagnostic applications. SELEX identifies nucleic acid ligands that are able to bind targets with high affinity and with surprising specificity. These characteristics are, of course, the desired properties one skilled in the art would seek in a diagnostic ligand.

The nucleic acid ligands of the present invention may be routinely adapted for diagnostic purposes according to any number of techniques employed by those skilled in the art or by the methods described in PCT/US98/00589 (WO 98/30720). Diagnostic agents need only be able to allow the user to identify the presence of a given target at a particular locale or concentration. Simply the ability to form binding pairs with the target may be sufficient to trigger a positive signal for diagnostic purposes. Those skilled in the art would also be able to adapt any nucleic acid ligand by procedures known in the art to incorporate a labeling tag in order to track the presence of such ligand. Such a tag could

16

be used in a number of diagnostic procedures. The nucleic acid ligands to TGFβ2
described herein may specifically be used for identification of the TGFβ2 protein.

SELEX provides high affinity ligands of a target molecule. This represents a
singular achievement that is unprecedented in the field of nucleic acids research. The
5   present invention applies the SELEX procedure to the specific target of TGFβ1. In the
Example section below, the experimental parameters used to isolate and identify the
nucleic acid ligands to TGFβ2 are described.

In order to produce nucleic acids desirable for use as a pharmaceutical, it is
preferred that the nucleic acid ligand: 1) binds to the target in a manner capable of
10   achieving the desired effect on the target; 2) be as small as possible to obtain the desired
effect; 3) be as stable as possible; and 4) be a specific ligand to the chosen target. In most
situations, it is preferred that the nucleic acid ligand have the highest possible affinity to
the target.

In co-pending and commonly assigned U.S. Patent No. 5,496,938, ('938 patent),
15   methods are described for obtaining improved nucleic acid ligands after SELEX has been
performed. The '938 patent, entitled "Nucleic Acid Ligands to HIV-RT and HIV-1 Rev,"
is specifically incorporated herein by reference in its entirety.

In the present invention, SELEX experiments were performed in order to identify
RNA ligands with specific high affinity for TGFβ2 from degenerate libraries containing
20   33, 34 or 40 random positions (33N, 34N or 40N) (**Table 1**). This invention includes the
specific RNA ligands to TGFβ2 shown in **Tables 5, 7, 8, 11, 13, 14, 16-19** and **Figure 9**
(SEQ ID NOS:21-108 and 128-193), identified by the methods described in Example 1.
This invention further includes RNA ligands to TGFβ2 which inhibit TGFβ2 function,
presumably by inhibiting the interaction of TGFβ2 with its receptor. The scope of the
25   ligands covered by this invention extends to all nucleic acid ligands of TGFβ2, modified
and unmodified, identified according to the SELEX procedure. More specifically, this
invention includes nucleic acid sequences that are substantially homologous to the ligands
shown in **Tables 5, 7, 8, 11, 13, 14, 16-19** and **Figure 9** (SEQ ID NOS:21-108 and 128-
193). By substantially homologous it is meant a degree of primary sequence homology in
30   excess of 70%, most preferably in excess of 80%, and even more preferably in excess of
90%, 95% or 99%. The percentage of homology as described herein is calculated as the

percentage of nucleotides found in the smaller of the two sequences which align with
identical nucleotide residues in the sequence being compared when 1 gap in a length of 10
nucleotides may be introduced to assist in that alignment. A review of the sequence
homologies of the ligands of TGFβ2, shown in **Tables 5, 7, 8, 11, 13, 14, 16-19** and

5   **Figure 9** (SEQ ID NOS:21-108 and 128-193) shows that some sequences with little or no
primary homology may have substantially the same ability to bind TGFβ2. For these
reasons, this invention also includes nucleic acid ligands that have substantially the same
structure and ability to bind TGFβ2 as the nucleic acid ligands shown in **Tables 5, 7, 8, 11,
13, 14, 16-19** and **Figure 9** (SEQ ID NOS:21-108 and 128-193). Substantially the same

10   ability to bind TGFβ2 means that the affinity is within one or two orders of magnitude of
the affinity of the ligands described herein. It is well within the skill of those of ordinary
skill in the art to determine whether a given sequence --substantially homologous to those
specifically described herein-- has substantially the same ability to bind TGFβ.

       This invention also includes nucleic acid ligands that have substantially the same

15   postulated structure or structural motifs. Substantially the same structure or structural
motifs can be postulated by sequence alignment using the Zukerfold program (see Zuker
(1989) Science 244:48-52). As would be known in the art, other computer programs can
be used for predicting secondary structure and structural motifs. Substantially the same
structure or structural motif of nucleic acid ligands in solution or as a bound structure can

20   also be postulated using NMR or other techniques as would be known in the art.

       One potential problem encountered in the therapeutic, prophylactic and *in vivo*
diagnostic use of nucleic acids is that oligonucleotides in their phosphodiester form may
be quickly degraded in body fluids by intracellular and extracellular enzymes such as
endonucleases and exonucleases before the desired effect is manifest. Certain chemical

25   modifications of the nucleic acid ligand can be made to increase the *in vivo* stability of the
nucleic acid ligand or to enhance or to mediate the delivery of the nucleic acid ligand.
See, e.g., U.S. Patent Application Serial No. 08/117,991, filed September 8, 1993, entitled
"High Affinity Nucleic Acid Ligands Containing Modified Nucleotides," now abandoned
and U.S. Patent No. 6,011,020, entitled "Nucleic Acid Ligand Complexes," which are

30   specifically incorporated herein by reference in their entirety. Modifications of the nucleic
acid ligands contemplated in this invention include, but are not limited to, those which

18

provide other chemical groups that incorporate additional charge, polarizability, hydrophobicity, hydrogen bonding, electrostatic interaction, and fluxionality to the nucleic acid ligand bases or to the nucleic acid ligand as a whole. Such modifications include, but are not limited to, 2'-position sugar modifications, 5-position pyrimidine modifications, 8-

5   position purine modifications, modifications at exocyclic amines, substitution of 4-thiouridine, substitution of 5-bromo or 5-iodo-uracil, backbone modifications, phosphorothioate or alkyl phosphate modifications, methylations, unusual base-pairing combinations such as the isobases isocytidine and isoguanidine and the like. Modifications can also include 3' and 5' modifications such as capping.

10   Where the nucleic acid ligands are derived by the SELEX method, the modifications can be pre- or post- SELEX modifications. Pre-SELEX modifications yield nucleic acid ligands with both specificity for their SELEX target and improved *in vivo* stability. Post-SELEX modifications made to 2'-OH nucleic acid ligands can result in improved *in vivo* stability without adversely affecting the binding capacity of the nucleic

15   acid ligand. The preferred modifications of the nucleic acid ligands of the subject invention are 5' and 3' phosphorothioate capping and/or 3'-3' inverted phosphodiester linkage at the 3' end. In one preferred embodiment, the preferred modification of the nucleic acid ligand is a 3'-3' inverted phosphodiester linkage at the 3' end. Additional 2'-fluoro (2'-F) and/or 2'-amino (2'-NH$_2$) and/or 2'-O methyl (2'-OMe and/or 2'-OCH$_3$) modification of some or all of

20   the nucleotides is preferred. Described herein are nucleic acid ligands that were 2'-F modified and incorporated into the SELEX process. Also described herein are nucleic acid ligands that were 2'-OCH$_3$ modified after the SELEX process was performed.

Other modifications are known to one of ordinary skill in the art. Such modifications may be made post-SELEX (modification of previously identified

25   unmodified ligands) or by incorporation into the SELEX process.

As described above, because of their ability to selectively bind TGFβ2, the nucleic acid ligands to TGFβ2 described herein are useful as pharmaceuticals. This invention, therefore, also includes a method for treating TGFβ2-mediated pathological conditions by administration of a nucleic acid ligand capable of binding to TGFβ2.

30   Therapeutic compositions of the nucleic acid ligands may be administered parenterally by injection, although other effective administration forms, such as

19

intraarticular injection, inhalant mists, orally active formulations, transdermal iontophoresis or suppositories, are also envisioned. One preferred carrier is physiological saline solution, but it is contemplated that other pharmaceutically acceptable carriers may also be used. In one preferred embodiment, it is envisioned that the carrier and the ligand

5 constitute a physiologically-compatible, slow release formulation. The primary solvent in such a carrier may be either aqueous or non-aqueous in nature. In addition, the carrier may contain other pharmacologically-acceptable excipients for modifying or maintaining the pH, osmolarity, viscosity, clarity, color, sterility, stability, rate of dissolution, or odor of the formulation. Similarly, the carrier may contain still other pharmacologically-

10 acceptable excipients for modifying or maintaining the stability, rate of dissolution, release, or absorption of the ligand. Such excipients are those substances usually and customarily employed to formulate dosages for parental administration in either unit dose or multi-dose form.

Once the therapeutic composition has been formulated, it may be stored in sterile

15 vials as a solution, suspension, gel, emulsion, solid, or dehydrated or lyophilized powder. Such formulations may be stored either in a ready to use form or requiring reconstitution immediately prior to administration. The manner of administering formulations containing nucleic acid ligands for systemic delivery may be via subcutaneous, intramuscular, intravenous, intranasal or vaginal or rectal suppository.

20 The following Examples are provided to explain and illustrate the present invention and are not intended to be limiting of the invention. Example 1 describes the various materials and experimental procedures used in Examples 2-5. Example 2 describes the isolation and characteristics of nucleic acid ligands that bind human TGFβ2. Example 3 describes the nucleic acid ligands isolated by the SELEX method using a biased round O library, the

25 sequences of TGFβ2 nucleic acid ligands isolated from the biased SELEX process, and the binding of nucleic acid ligands isolated from the biased SELEX process. Example 4 describes substitutions of 2'-OH purines with 2'-OCH₃ purines in NX22284 (SEQ ID NO:115) and NX22385 (SEQ ID NO:189). Example 5 describes the pharmacokinetic properties of NX22323 (SEQ ID NO:121). Example 6 describes 2'-OMe modification of

30 lead TGFβ1 truncate ligand CD70 (SEQ ID NO:216). Example 7 describes a Mink Lung Epithelial Cell (MLEC) PAI Luciferase Assay of TGFβ aptamers to TGFβ1 and TGFβ2.

EXAMPLES

Example 1. Experimental Procedures

Materials

5   Monoclonal and polyclonal antibodies that recognize human TGFβ1, TGFβ2 or

TGFβ3 were purchased from R&D Systems, Inc. (Minneapolis, MN). DNA

oligonucleotides were purchased from Operon, Inc. (Alameda, CA) or Oligos, Etc.

(Redding Center, CT). The BIAcore 2000 and IAsys *plus* instruments are products of

Biacore, Inc. (Paramus, NJ) and Affinity Sensors, Inc. (Cambridge, U. K.), respectively.

10   Nitrocellulose filters and filtering manifolds were obtained from Millipore (Bedford, MA).

Mink lung epithelial cells (#CCL64) were purchased from the American Type Culture

Collection (Rockville, MD). The cloning vectors pCR-Script and pUC9 were obtained in-

house or from Stratagene, Inc. (La Jolla, CA) or Life Technologies, Inc. (Gaithersburg,

MD), respectively. *E coli* strains were obtained from Stratagene. The QIAprep spin

15   miniprep kit was from QIAgen, Inc. (Chatsworth, CA). The Big Dye sequencing kit and

model 377 sequencer can be purchased from Applied Biosystems (Foster City, CA). T7

RNA polymerase and *Thermus aquaticus* DNA polymerase were purchased from Enzyco,

Inc. (Denver, CO) and Perkin Elmer (Norwalk, CT), respectively. All restriction enzymes

were purchased from New England Biolabs. *E. coli* RNase H was obtained from

20   Boehringer Mannheim. All synthetic nucleic acids with a name that begins with "NX"

were synthesized at NeXstar Pharmaceuticals, Inc. (Boulder, CO) using an ABI model 394

DNA/RNA synthesizer (Applied Biosystems). Yeast tRNA (type X-SA) and porcine

intestinal mucosca-derived heparin (molecular weight 5000), were purchased from Sigma

(St. Louis, MO) and Calbiochem (La Jolla, CA), respectively.

25   **Preparation of round 0 nucleic acid library**

          The initial (round 0) library of ribonucleic acid molecules that was used to isolate

TGFβ2 nucleic acid ligands was generated as follows. Two DNA oligonucleotides (**40 N7**

round 0 (R0) DNA template and 5'N7 primer) were annealed and filled in with Klenow

to produce a **40 N7 R0 DNA transcription template (Table 1)**. This template was

30   transcribed using T7 RNA polymerase, 3 mM 2-F uridine and cytosine, 1 mM 2'-OH

guanosine and adenine, and α ^{32}P-ATP as described in (Fitzwater and Polisky (1996)

Meth. Enz. 267:275-301). This resulted in a **R0 40N7 nucleic acid** pool with the

following sequence which has 5' and 3' "fixed" regions and a 40 base long random

sequence region:

5   5'-GGGAGGACGAUGCGG-40N-CAGACGACUCGCCCGA-3') **R0 40N7 nucleic acid**
             5' fixed region   random region   3' fixed region   (SEQ ID NO:6)
        A=2'-OH A; C=2'-F C; G=2'-OH G; U=2'-F U   **(Table 1)**

Spot SELEX

10   Spot SELEX was performed as described in U.S. Patent No. 5,972,599, entitled

"High Affinity Nucleic Acid Ligands of Cytokines," which is hereby incorporated by

reference in its entirety, using nucleic acid that was internally labeled using α-^{32}P ATP.

The conditions and progress of this SELEX experiment are summarized in **Table 3**.

Briefly, human TGFβ2 (or no protein) was applied to a 13 mm diameter nitrocellulose

15   filter and allowed to absorb but not completely dry. The filter was incubated with RNA in

Dulbecco's phosphate-buffered saline, 1 mM MgCl$_2$ and then washed as summarized in

Table 3. Filter-bound and protein-bound nucleic acid was visualized and quantitated on

an Instant *Imager* (Packard Instrument Co., Downers Grove, IL) and the protein-bound

nucleic acid was eluted in 50% phenol, 4 M urea for 45 minutes at 65°C. Eluted nucleic

20   acid was ethanol precipitated and then reverse transcribed using avian myeloblastosis virus

reverse transcriptase and subjected to the polymerase chain reaction (PCR) using 5'N7 and

3'N7 primers for 15 cycles. This resulting transcription template was transcribed with T7

RNA polymerase in the presence of 2'-F pyrimidine nucleotides, 2'-OH purine

ribonucleotides and α^{32}P-ATP, and carried to the next spot round. The pool from the first

25   spot round was also transcribed as above in the absence of α-^{32}P-ATP for use in round 2 of

the surface plasmon resonance biosensor SELEX.

Surface plasmon resonance biosensor SELEX

Rounds 2-spr through 9-spr were done using surface plasmon resonance biosensor

technology on a BIAcore model 2000 instrument. For this experiment 1XDPBS, 1 mM

30   MgCl$_2$, 0.005% P20 surfactant (cat#BR-1000-54, Biacore, Inc., Piscataway, NJ) was used

as the running buffer. TGFβ2 was amine coupled onto a CM5 BIACORE chip (Biacore,

Inc., Piscataway, NJ) using the Biacore amine coupling kit (cat#BR-1000-50, Biacore,

Inc., Piscataway, NJ) per manufacturer's instructions. Briefly, TGFβ2 aliquots (3 μL, in 4 mM HCl at 100 μg/mL) were diluted in 30 μL of 10 mM CH_3COONa, pH 5.0 and injected on an EDC-NHS activated chip at 25°C, 5 μL /min, in different volumes to achieve different loading levels, as measured in response units (RU). Following coupling, the chip

5   was washed with 3M NaCl for about 1.5 minutes at 10 μL/min. Under these experimental conditions, TGFβ2 loading of 15RU/μL could be achieved. TGFβ2 was loaded in flow cells 1, 2, and 3, while flow cell 4 was kept blank for control and background subtractions. Before use, the chip was tested for activity by testing binding of LAP and or soluble receptor III (R&D Systems, Minneapolis, MN) at 37°C. At the end of each test

10   injections the chip was regenerated using a 1 minute wash with 10 mM NaOH. For SELEX rounds, RNA pools, generated by *in vitro* transcription without any labeled nucleotides, were in running buffer and were injected over the TGFβ2 loaded CM5 chips at 5 μL/min at 37°C. The concentration and volume of the RNA pools used at each round are as shown in **Table 4**. At each round the RNA pools were applied in 40 μL injections

15   and each injection cycle was followed by a dissociation phase where the chip was washed with DPBS, 1mM $MgCl_2$ at 20 μL/min while three 100 μL fractions (5 minutes each) were collected. Following the last injection-dissociation cycle, the chip was treated with 0.25% SDS and the eluted RNA was collected as the final fraction. The third fractions of each injection cycle and the SDS elution were pooled and amplified by RT/PCR to generate the

20   template pool for the next SELEX round.

Resonant Mirror Optical Biosensor SELEX

    Rounds 10-rm through 13-rm were done using an IASYS *plus* resonant mirror optical biosensor instrument. Round 9-spr from the surface plasmon resonance SELEX was used as the starting material. For this experiment, 1XDPBS, 1 mM $MgCl_2$, 0.005%

25   P20 surfactant (cat#BR-1000-54, Biacore, Inc., Piscataway, NJ) were used as the running buffer. TGFβ2 was amine coupled onto a CMD IASYS cuvette (Affinity Sensors, Cambridge, UK) according to the manufacturer's protocol. Briefly, the CMD cuvette was activated with 0.2 M EDC, 0.05 M NHS for 10 minutes, and TGFβ2 was coupled by injection 35 μL of 0.4 μM TGFß2, 10 mM CH_3COONa, pH 5.5 in 35 μL of 10mM

30   CH_3COONa, pH 5.5. The coupling reaction was at 25°C for about 10 minutes and resulted in about 2,000 Arcsec of signal. Unreacted sites were capped by exposing the cuvette in

23

1M ethanolamine for 1-2 minutes. Following coupling and capping the cuvette was

exposed to 3M NaCl for 1-2 minutes and was ready for use. The cuvettes were routinely

tested for activity by measuring binding of LAP and or soluble receptor III (R&D

Systems, Minneapolis, MN) at 37°C. At the end of each test injection the chip was

5   regenerated using 1 minute wash with 50 mM NaCO₃. For SELEX rounds, RNA pools,

generated by in vitro transcription without any labeled nucleotides, were in running buffer.

They were injected in the TGFβ2 loaded CMD cuvette and incubated for 27-60 minutes

(**Table 6**) under 100% steering at 37°C. Following binding, the RNA was replaced with

buffer and bound RNA was observed to dissociate from the cuvette surface. Dissociation

10   was allowed for 30-150 minutes (**Table 6**) at 37°C while the buffer was exchanged several

times to avoid evaporation. Following dissociation, the remaining RNA was eluded with

H₂O or 0.25% SDS and the RNA was amplified as above and carried to the next SELEX

round.

SELEX using filter partitioning and polyanion competition

15   For rounds 9b through 22a, SELEX using filter partitioning was performed

essentially as described in (Fitzwater and Polisky (1996) Meth. Enz. 267:275-301) except

that 1) heparin or yeast tRNA was included to compete off ligands that bound

nonspecifically, 2) the binding buffer was HBSMCK (50 mM HEPES, pH 7.4, 140 mM

NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 3 mM KCl), 3) extensive efforts were undertaken to

20   reduce filter binding sequences (preadsorbtion of nucleic acid onto filters after elution and

transcription, blocking of filters with tRNA and bovine serum albumin prior to

partitioning, addition of 0.5 M urea to the wash buffer) and 4) the transcripts were initiated

with a 5:1 molar mixture of guanosine:2'-fluoro-nucleotides. Initiation with guanosine

allows nucleic acids to be used in SELEX or bioactivity assays without radiolabeling and

25   alleviates a phosphatase step if the nucleic acid is to be 5'-end radiolabeled for binding

studies.

        Round 8-spr from the surface plasmon resonance SELEX was used as the starting

material. From rounds 9b to 14i, the SELEX process was performed using protein-excess

conditions. The concentrations of nucleic acid and protein were equimolar in round 15c.

30   Nucleic acid-excess conditions were used from rounds 16a to 22a (**Table 2**). Competitors

(yeast tRNA and heparin) were used from rounds 9b to 14i. Filters were washed with 10-

24

15 mL HBSMCK buffer from rounds 9b to 12d and increasing amounts (5-50 mL) of

HBSMCK, 0.5 M urea from rounds 13i to 22a.

Sequencing of nucleic acid ligand pools

　　　　　Nucleic acid pools were sequenced as described in (Fitzwater and Polisky (1996)

5　　Meth. Enz. 267:275-301).

Screening nucleic acid ligand pools using ligand-specific reverse transcription-PCR

　　　　　Nucleic acids from the various pools was reverse transcribed with clone-"specific"

primers (ML-85 (SEQ ID NO:16) for ligand 14i-1 and **ML-81 (SEQ ID NO:18)** for

ligand 21a-21) for 12, 15 or 18 cycles. Mixtures of pure nucleic acid ligands and round 0

10　　40N7 nucleic acid that contained 10%, 3%, 1%, 0.3% or 0.1% ligand were processed in

the same manner and served to quantitate signals from RT-PCR of the nucleic acid pools.

Cloning, screening and sequencing of nucleic acid ligands

　　　　　Nucleic acid ligands were cloned using two methods. In one method the ligands

were directly cloned into pCR-Script according to the manufacturer's instructions and

15　　transformed into *E. coli* strain XL-1 Blue MRF' Kan. In the other method the double-

stranded DNA transcription template was amplified by PCR using primers **ML-34 (SEQ**

ID NO:11) and **ML-78 (SEQ ID NO:12)**, digested with BamHI and EcoRI restriction

enzymes, and cloned into BamHI and EcoRI-digested pUC9. The ligation was

transformed into *E. coli* strain DH5α. Colonies were selected on ampicillin plates and

20　　screened for inserts by PCR using vector-specific primers **(RSP1 (SEQ ID NO:13) and**

FSP2 (SEQ ID NO:14)). Typically 90%-100% of the clones had inserts. Some colonies

or nucleic acid pools were also screened using 14i-1, 21a-4, or 21a-21 ligand-specific

primers **(ML-79 (SEQ ID NO:17), ML-81 (SEQ ID NO:18)**, and **ML-85 (SEQ ID**

NO:16), respectively) in an attempt to identify clones that were different from those

25　　already isolated.

　　　　　Plasmid minipreps from the transformants were prepared using the QIAprep spin

miniprep kit (QIAGEN, Inc., Valencia, CA) or PERFECT prep plasmid DNA kit (5'3',

Inc., Boulder, CO). Sequencing reactions were performed with the Big Dye kit and a

sequencing primer **(RSP2)**. The sequencing products were analyzed on an ABI model 377

30　　sequencer.

Nucleic acid ligand boundaries

The boundaries (5' and 3' end) of the smallest ligand that can bind TGFβ2 was determined essentially as described in (Fitzwater and Polisky (1996) Meth. Enz. 267:275-301). The protein concentrations used were 0, 1 nM, and 10 nM and the nucleic acid

5   /protein ratio was 1. The binding buffer used in this experiment was HBSMC, 0.01% HSA. Binding reactions were incubated at 37°C for 30 minutes, filtered through 0.45 μm, nitrocellulose filters (15 mm),and then washed with 15 mL HBSMC. The RNA was recovered by phenol-urea extraction, eluted RNA was ethanol precipitated in the presence of glycogen, resuspended in H_2O, supplemented with equal volume 2X formamide dye,

10   and analyzed on 8% acrylamide, 8M urea sequencing gels. Truncated RNAs that were bound to TGFβ2 were visualized and developed on a FUJIX BAS1000 phosporimager (FUJI Medical Systems, USA).

Nucleic acid ligand truncation

Truncated versions of full length nucleic acid ligands were generated in three ways.

15   In one method, *E. coli* RNase H and hybrid 2'-OCH₃ RNA/DNA oligonucleotides (**5'N7 cleave, 3'N7 cleave; Table 1**) were used to cleave nucleic acids at a specific site. Truncation SELEX is described in U.S. Patent Application Serial No. 09/275,850, filed March 24, 1999, entitled "Truncation SELEX Method," which is hereby incorporated by reference in its entirety. In a second method, overlapping DNA oligonucleotides encoding

20   the desired ligand sequence were annealed, extended by Klenow DNA polymerase, and then transcribed. In a third method, ligands were chemically synthesized with the desired sequence.

Binding of nucleic acid ligands to human TGFβ's

The binding activity of individual ligands was determined by measuring the

25   equilibrium dissociation constants using nitrocellulose partitioning of labeled RNA as a function of protein concentration. RNA was body-labeled or guanylated and then 5'-end labeled with γ-^{32}P ATP and T4 polynucleotide kinase. Binding reactions were set at various protein concentrations (typically varied in either 3-fold or 10-fold increments) while maintaining the labeled RNA concentration constant at less than 0.1 nM, and

30   incubated at 37°C for 10 minutes. Protein-RNA complexes were partitioned away from uncomplexed RNA, by filtering the binding reactions through a nitrocellulose/cellulose

26

acetated mixed matrix (0.45 μm pore size filter disks, type HA; Millipore, Co., Bedford,

MA). For filtration, the filters were placed onto a vacuum manifold (12-well, Millipore, or

96-well BRL) and wetted by aspirating 1-5 mL of binding buffer. The binding reactions

were aspirated through the filters, washed with 1-5 mL of binding buffer and counted in a

5   scintillation counter (Beckmann).

To obtain the monophasic equilibrium dissociation constants of RNA ligands to

hTGFβ2 the binding reaction:

$$K_D$$
$$R:P \rightarrow R+P$$

10
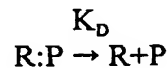
wherein R=RNA; P=Protein and K_D=dissociation constant is converted into an equation

for the fraction of RNA bound at equilibrium:

$$q=(f/2R_T)(P_T+R_T+K_D-((P_T + R_T + K_D)^2-4\ P_T\ R_T)^{1/2})$$

wherein q=fraction of RNA bound; P_T =total protein concentration; R_T =total RNA

15   concentration and f=retention efficiency of RNA-protein complexes. The average

retention efficiency for RNA-TGFβ2 complexes on nitrocellulose filters is 0.3-0.8. Kd

values were obtained by least square fitting of the data points using the software

Kaleidagraph (Synergy Software, Reading, PA).

Competition between ligands

20   ^{32}P-labeled test ligands at a concentration of 1 nM were mixed with increasing

concentrations of unlabeled NX22283 (SEQ ID NO:114). Then, an amount of TGFβ2

estimated to be near the Kd of the test ligands was added (1 nM for NX22283, 1 nM for

21a-21, 3 nM for 21a-4, and 10 nM for 14i-1). The reactions were incubated, filtered,

washed, and counted as for a binding reaction.

25   **Off-rate of NX22283**

1 nM ^{32}P-labeled NX22283 was mixed with 10 nM TGFβ2, incubated for 5

minutes to allow the protein to bind to the nucleic acid, and then a 1000-fold excess (1

μM) of unlabeled NX22283 was added. At various time points the reactions were filtered

and washed to measure the amount of ^{32}P-labeled NX22283 that remained bound.

30   **Biased SELEX**

A library of sequences was constructed based on the sequence of the 34-mer

truncate (NX22284 (SEQ ID NO:115)) of nucleic acid ligand 21a-21. The sequence of the

27

DNA template (34N7.21a-21 (SEQ ID NO:7)) is shown in **Table 1**. The randomized region is 34 bases long. At each position the randomized region consists of 62.5% of the NX22284 sequence and 12.5% of the other 3 nucleotides. Thus the randomized region is mutagenized at each position (37.5%), but at the same time is biased toward the sequence

5   of NX22284. The fixed regions (5'N7, 3'N7) were the same as used for the primary SELEX.

To generate **34N7.21a-21 round 0 nucleic acid,** the DNA template was amplified by PCR using the **5'N7** (SEQ ID NO:2) **and 3'N7** (SEQ ID NO:3) primers (**Table 1**). This PCR product was transcribed as described above in the filter partitioning SELEX

10   section. This resulted in a **34N7.21a-21 round 0 nucleic acid** pool with the sequence shown in **Table 1** (SEQ ID NO:10).

Filter partitioning as described above and in Fitzwater and Polisky (1996) (Meth. Enz. 267:275-301) with no competitors was used to enrich nucleic acids ligands that bound to human TGFβ2 the best. The protein concentration was reduced from ~150-300

15   nM to 50 pM. The nucleic acid concentration was reduced from 1 μM to 1 nM. The nucleic acid/protein ratio ranged from 0.25 to 125. The round 5a pool of ligands was cloned into pUC9 and sequenced as described above.

Bioactivity of TGFβ2 nucleic acid ligands

The bioactivity of TGFβ2 nucleic acid ligands was measured with mink lung

20   epithelial cells. Proliferation of these cells is inhibited by TGFβ2. Human TGFβ2 was titrated on the cells and ^{3}H-thymidine incorporation was measured. The point at which ^{3}H-thymidine incorporation by the cells was inhibited by 90-100 % was determined (typically 1-4 pM). This inhibitory amount of TGFβ2 along with varying amounts of nucleic acid ligand (typically 0.3 or 1 nM to 1 or 3 μM, in 3 fold increments) was used. Typically,

25   cells were plated at 10E5/mL in 96-well plates in 100 μL MEM, 10mM HEPES pH 7.4, 0.2% FBS. Following a 4 hour incubation at 37°C, when cells were well attached to the well surface, TGFβ2 was added at 1-4 pM with or without nucleic acid ligands as follows: the ligands were diluted across the 96 well plate in 3-fold dilution steps and then TGFβ2 was added at 1-4 pM to all wells except controls. The cells were incubated for 16-18

30   hours prior to addition of ^{3}H-thymidine, and then incubation was continued for 20 additional hours following ^{3}H-thymidine addition at 0.25 μCi per well. After incubation,

28

the cells were lysed with 1% Triton X-100 and harvested onto GF/B filter plates in a Packard 96 well plate harvester, and ^{3}H-thymidine incorporation in cellular DNA was quantitated by scintillation counting in MicroScint (Packard, Mariden, CT) in a Packard Top-Count. Data were plotted as % of maximum ^{3}H-thymidine incorporation vs RNA

5   concentration, and were fitted by the software Kaleidagraph (Synergy Software, Reading, PA) to the equation $m3*(m0+m1+(m2)-((m0+m1+(m2))*(m0+m1+(m2))-4*(m0)*((m2)))^0.5)/(2*(m2))$; where m0 is the concentration of competitor RNA; m1 is the IC50, m2 is the concentration of TGFβ2, and m3 is the plateau value of the fraction of maximum ^{3}H-thymidine incorporation. K_i values were determined from IC_{50} values according to the

10   equation $K_i=IC_{50}/(1+([T]/K_{dT})$, where [T] is the molar concentration of TGFβ2 present in the assay and K_{dT} is the concentration of TGFβ2 causing 50% inhibition of MLEC proliferation as determined by TGFβ2 titration experiments. This assay was also used to determine the isotype specificity of RNA ligands where the three TGFβ isotypes were independently used as inhibitors of MLEC replication.

15   **Pharmacokinetic properties of NX22323 (SEQ ID NO:121)**

The pharmacokinetic properties of TGFβ2 ligand NX22323 were determined in Sprague-Dawley rats. NX22323 was suspended in sterile PBS and stored at ≤-20°C. Prior to animal dosing NX22323 was diluted with sterile PBS, to a final concentration of 0.925 mg/mL (18 μM, based on the oligonucleotide molecular weight and the ultraviolet

20   absorption at 260 nm with an extinction coefficient of 0.037 mg of oligo/mL). Sprague-Dawley rats (n = 2) were administered a single dose of NX22323 by intravenous bolus injection through the tail vein. Blood samples (approximately 400 μL) were obtained by venipuncture under isofluorane anesthesia and placed in EDTA-containing tubes. The EDTA-treated blood samples were immediately processed by centrifugation to obtain

25   plasma and stored frozen ≤-20°C. Time points for blood sample collection ranged from 5 to 2880 minutes.

Standards and quality control samples prepared in blank rat plasma and plasma samples were analyzed by a double hybridization assay. To prepare plasma samples for hybridization analysis, 25 μL of plasma sample (or a dilution in plasma of the sample) was

30   added to 100 μL of 4 x SSC, 0.5 % sarkosyl. A 25 μL aliquot was then mixed with 25 μL of 4 x SSC, 0.5 % sarkosyl containing 24 μM capture oligonucleotide conjugated to

magnetic beads and 28 μM detect oligonucleotide conjugated to biotin in a covered 96-
well microtiter plates. The mixture was allowed to incubate at 45°C. for 1 hour. Unbound
oligonucleotide was removed and 0. 1 ng streptavidin alkaline phosphatase/μL NTT
Buffer (0.8 M NaCl, 20 mM Tris pH 7.5, 0.5% Tween 20) added to each well followed by

5   a 30 minute incubation at room temperature. The streptavidin alkaline phosphatase was
removed and the plate was washed twice with 200 μL NTT Buffer. The NTT Buffer was
removed and replaced with 50 μL DEA buffer (0.02% NaN$_3$, 1 mM MgCl$_2$, 1%
diethanolamine (Tropix, Inc., Bedford, MA), pH 10) and 34 μL/mL 25 mM
chemiluminescent substrate for alkaline phosphatase (Tropix, Inc., Bedford, MA), and

10   20% Sapphire chemiluminescence amplifier (Tropix, Inc., Bedford, MA) in DEA buffer
(50 μL/well) was added. The plate was incubated for 20 minutes at room temperature and
read on a luminometer. A standard curve of NX22323 was fit using a variable slope
sigmoidal dose response non linear regression equation (PRISM, version 2.00, GraphPad,
San Diego, CA). Sample and quality control concentrations were extrapolated from the

15   standard curve and corrected for dilution.

The average plasma concentration at each time point was calculated and utilized in
the pharmacokinetic analysis. Both noncompartmental and compartmental
pharmacokinetic analysis were carried out using WinNonlin version 1.5 (Scientific
Consulting, Inc.). In the noncompartmental analysis, the following parameters were

20   calculated; the maximum concentration extrapolated at zero time (Cmax), the area under
the curve from zero to the last time point (AUClast), the area under the curve from zero to
infinite time (AUCINF), the terminal phase half life (Beta t1/2), the clearance rate (Cl), the
mean residence time calculated from zero to infinite time (MRTINF), the volume of
distribution at steady state (Vss), and the volume of distribution during elimination (Vz).

25   In the case of compartmental analysis, the following parameters were calculated based on
the minimum number of monoexponential equations to adequately fit the data: the
maximum concentration extrapolated at zero time (Cmax), the area under the curve from
zero to infinite time (AUCINF), the distribution phase half life (Alpha t1/2), terminal
phase half life (Beta t1/2), the exponential constant for the distribution phase (A), the

30   exponential constant for the terminal phase (B), the clearance rate (Cl), the mean residence

observed on the biosensor in round (Rd) 6, increased up to round 9, and then decreased in rounds 10 and 11. **Figure 3** shows sensograms with 0, 4-spr, 6-spr, 8-spr, and 9.-spr. **Figure 4** shows typical filter binding curves, in the presence and absence of competitor tRNA, with representative pools from the spr SELEX and from these data it seems that

5   round 9 binds in a biphasic manner with a high affinity and low affinity K_d of 30 and 160 nM, respectively.

In bioactivity assays the K_i of the round 0 pool was about 2.6 μM and the K_i of the round 9-spr pool was about 711 nM (see below). Sequence analysis of representative pools indicated that such pools maintained significant complexity up to round 8 while after

10   round 9 such pools were strongly biased towards a single sequence.

Round 8-spr was cloned and sequenced. A total of 69 clones representing 51 different sequences were analyzed. Four sequences (Nos 8.2 (SEQ ID NO:22), 8.3 (SEQ ID NO:23), 8.9 (SEQ ID NO:27), and 8.48 (SEQ ID NO:54); see **Table 5**) were represented more than once and accounted for 21 of the 69 clones. All four of these

15   sequences bound TGFβ2 and were 2 or 3 base variants of a clone (14i-1) isolated from the filter SELEX (see below). Twenty three other sequences were nonbinding or filter-binding sequences (see **Table 5**) and 25 clones were not tested for binding.

Resonant mirror (rm) optical biosensor SELEX

Since the affinity of nucleic acid pools selected on the surface plasmon resonance

20   biosensor peaked at round 9-spr, resonant mirror (rm) optical biosensor technology was tested to determine if it could advance the affinity of nucleic acid ligands any further. Resonant mirror optical biosensor technology offers many of the same advantage as surface plasmon biosensor technology, but in addition the binding is done in a cuvette under equilibrium conditions rather than over the surface of a chip under flow conditions.

25   Within the cuvette the binding can be extended for long time periods. Therefore, the nucleic acid/protein binding reaction can be more stringent and selective.

For resonant mirror SELEX, TGFβ2 was coupled to two biosensor cuvettes using amine coupling chemistry. In one cuvette the TGFβ2 was inactivated by SDS denaturation and this cuvette served to assess background. The other cuvette containing

30   active TGFβ2 was used for the SELEX. Beginning with round 9-spr, five rounds were

done using resonant mirror optical biosensor technology. The conditions used for and the results of the resonant mirror SELEX are shown in **Table 6**.

Biosensor signals were observed for each round. The binding of the nucleic acid pools from rounds 10-rm to 12-rm was assessed (**Table 6; Figure 5**). The pool K_d

5   improved modestly up to round 12-rm with no further improvement in the subsequent rounds. The round 12-rm pool binds biphasically with high and low affinity Kd of ~2nM and ~150nM, respectively.

In bioactivity assays the K_i of the round 13-rm pool was about 505 nM. Round 13-rm was chosen for subcloning and sequence analysis. Of 15 clones that were sequenced,

10   all 15 (**Table 7**) were 1 to 5 base variants of a clone (14i-1), which was originally isolated from the filter SELEX (see below).

Filter partitioning SELEX

Round 8-spr was used as the starting material for a filter SELEX. The properties of round 8-spr were studied and it was found that 1) a significant fraction bound to a

15   nitrocellulose filter (10%), 2) significant nucleic acid binding (defined here as signal/noise>2) to TGFβ2 was not detectable using nucleic acid-excess conditions, and 3) in the presence or absence of polyanionic competitors there was a significant decrease in the binding of round 0, but not round 8-spr to TGFβ2. These findings had implications that are addressed below.

20   Use of a competitor

The binding of round 8-spr nucleic acid to TGFβ2 in the presence of a polyanionic competitor (yeast tRNA) was studied at various ratios of competitor to nucleic acid. It was found that a 75,000 fold excess of tRNA over round 8-spr nucleic acid resulted in 50% inhibition of binding, whereas a 6,000 fold excess of tRNA over the round 0 nucleic acid

25   pool resulted in 50% inhibition of binding. Heparin also competed with RNA for binding to TGFβ2, but about 10-fold more heparin was needed to inhibit RNA binding to TGFβ2 to the same degree as that observed using tRNA. By including a 100,000-fold excess of yeast tRNA over RNA in a TGFβ2/RNA binding experiment, a 100-fold difference in binding between round 0 and round 8-spr was detected, whereas a 3-fold difference was

30   observed in the absence of any competitor. Thus, in the presence of an appropriate amount of competitor, the binding of selected nucleic acid pools is unaffected, whereas the binding

of round 0 nucleic acid is reduced substantially. When competitors are not included in studying the binding of TGFβ2 to nucleic acid the affinity of nucleic acids selected using the SELEX process can be grossly underestimated. In this regard TGFβ2 is similar to other "professional" nucleic acid binding proteins (e.g., restriction enzymes, polymerases,

5   transcription factors, etc.), in that it possesses both a low affinity, nonspecific and a high affinity, specific nucleic acid binding activity. The difference between these 2 binding modes can be revealed in the presence of competitors. Competitors are often used in the study of transcription factors. For example, it can be difficult to detect specific binding of a crude extract containing a transcription factor to oligonucleotides representing their

10   cognate site in gel-shift experiments, unless a competitor, such as poly [dI-dC] • poly [dI-dC], is included in the binding reaction.

Nonspecific binding can involve the binding of multiple proteins per nucleic acid, often at low affinity sites, giving a false appearance of high affinity. A protein can bind at multiple sites on a nucleic acid or protein aggregates may form on a single protein bound

15   to a nucleic acid. TGFβ2 is well known to be "sticky". In the absence of a competitor of nonspecific interactions, TGFβ2 may form large networks and complexes of nucleic acid and protein involving primarily nonspecific interactions. Gel shift analysis of TGFβ2, in the absence of competitor, supports these ideas because TGFβ2 does not form distinct (one to one) complexes with nucleic acid in gels, but instead either remains in the well at the

20   top of a gel or forms smears that may represent large heterogeneous nucleic acid/protein complexes.

Besides the implications for doing binding curves, the nucleic acid -binding properties of TGFβ2 may have implications for SELEX. For example the high level of nonspecific binding of nucleic acid by TGFβ2 may have interfered with previous SELEXs

25   by obscuring specific interactions or preventing the isolation of nucleic acid /protein complexes that involved only specific binding interactions. That is, if mixtures of specific and nonspecific nucleic acid interactions exist in nucleic acid/TGFβ2 complexes that form, then the selection for specific interactions may be difficult, if the nonspecific interactions are not eliminated. Lack of progress in some previous SELEX experiments may have

30   been due to efficient competition by the large excess ($>10^{12}$-10^{14}) of low affinity nucleic acids that contain nonspecific binding sites with a smaller number (~10-1000) of high

34

affinity nucleic acids that contain specific binding sites, especially in early rounds of SELEX.

<u>Use of protein-excess or nucleic acid-excess conditions</u>

Given the discussion above, a question arises as to which SELEX conditions are

5   better for a protein, such as TGFβ2, protein-excess or nucleic acid-excess. Protein-excess conditions may tend to encourage nonspecific interactions. However as long as the competitor/nucleic acid ratio is high enough to eliminate enough nonspecific interactions, but retain specific interactions, this may not be an issue. One advantage to using protein excess is the bound to background ratios are better and background is lower, which would

10   result in better levels of enrichment.

Nucleic acid-excess conditions may not discourage nonspecific interactions because within nucleic acid pools used for SELEX the ratio of nonspecific to specific binding nucleic acids (which is what is most important) would be the same no matter what the nucleic acid concentration is. In addition, nucleic acid-excess would reduce the

15   competitor/nucleic acid ratio which would tend to increase nonspecific interactions. As discussed above the ratio of tRNA to nucleic acid must be at least 100,000 in early rounds of the filter SELEX in order for affinity enrichment to be efficient. This can be technically difficult in early rounds of SELEX when the nucleic acid concentration is typically higher. One advantage to using excess nucleic acid is that more members of a given sequence

20   would be represented in a pool. However if there had been enough enrichment (e.g., using a method such as surface plasmon resonance SELEX) prior to filter SELEX there will probably be multiple representatives of a given sequence and this would not be an issue.

<u>Filter SELEX conditions</u>

The conditions used at each round of the filter SELEX are shown in **Table 15**.

25   Multiple conditions (up to 12) were used in each round varying nucleic acid/protein ratios, competitor/nucleic acid ratios, filter washing buffers, and filter washing volumes. Typically conditions that resulted in the lowest background (<1%) and a significant bound/background ratio (>2) were processed for the next round. Only data for SELEX rounds that were used in the next round are shown in **Table 15**.

30   The SELEX began by using an amount of tRNA competitor (100,000-fold excess) that was determined *in the SELEX reaction* to inhibit binding of round 8-spr to TGFβ2 by

about 60%. SELEX reactions with competitor were done for round 9b through 14i. The inclusion of tRNA in round 9 also dramatically reduced binding of round 8-spr nucleic acid to nitrocellulose filters from ~10-15% to ~1%. The higher the "background" binding is in a SELEX reaction, the lower the maximum possible enrichment. Thus, inclusion of

5   tRNA in the early rounds of the filter SELEX may have had a dual benefit. It not only may have eliminated nonspecific binding of TGFβ2 to nucleic acid, but also allowed more enrichment by reducing background. At round 15c lower affinity competitors were no longer effective at reducing binding of nucleic acid and were not used. This is presumably because the nucleic acid pool bound TGFβ2 with adequate specificity and affinity.

10   Therefore from round 16 to 22, the presumed specific nucleic acids were allowed to compete with each other by using more traditional nucleic acid -excess conditions.

        The background increased to unacceptable levels in rounds 15c and 16a. Gel shift partitioning was investigated as an alternative partitioning procedure at this point but did not work. By modifying the washing conditions the background was reduced to 0.2% in

15   round 17a. After round 18b it was possible to do SELEX rounds at protein concentrations below 1 nM and under nucleic acid-excess conditions. It was also found that nucleic acid concentrations above 1-5 nM helped to reduce background in some rounds.

        In summary, during the filter SELEX, the concentration of the protein was reduced 30,000-fold, from 300 nM in round 9b to 10 pM in round 22a. The background binding to

20   filters was reduced from 10% to 0.1%. Nucleic acid pools that bound to TGFβ2 only when protein-excess conditions (~100 protein/1 nucleic acid) were used were selected to bind under high nucleic acid/protein (>100/1) or competitor/nucleic acid (>10^7/1)-excess conditions.

Binding of nucleic acid pools from filter SELEX

25   The binding of TGFβ2 to selected nucleic acid pools improved steadily, but slowly and erratically. There was an improvement in the binding of round 10b (K_d=~100 nM) compared to the starting pool (round 8-spr; K_d=~500 nM). The affinity of round 11a was the same as 10b and that of round 12d improved modestly to ~40 nM. Rounds 13i and 15c bound TGFβ2 approximately the same (K_d=~30), while round 14i may have bound

30   worse (K_d=~75). Round 16a nucleic acid (K_d=~10 nM) bound slightly better than round 15c. There was ~2-fold improvement in affinity of the nucleic acid pool from rounds 16a

to 17a ($K_d = \sim 5$ nM). The K_d of round 18a nucleic acid (~ 5 nM) was equivalent to round 17a nucleic acid. There was another slight increase in affinity from round 18b to 19a ($K_d = \sim 2\text{-}3$ nM). The affinity of rounds 20a, 21a, and 22a plateaued at about 1 nM. The SELEX was stopped at round 22a because the bound to background ratio was below 2 and

5   it would have been technically difficult to reduce the protein concentration to 1-3 pM in round 23.

In summary the K_d improved from ~ 500 nM in round 9b to ~ 1 nM in round 21a, resulting in an overall improvement of ~ 500-fold in the filter SELEX and >10,000-fold in the entire SELEX. The average improvement per round was about 1.6-fold. This rate of

10   improvement is slow compared to an average SELEX experiment, which may take ~ 5 rounds using only surface plasmon resonance technology or ~ 10 rounds using only filter partitioning.

Inhibition of bioactivity by nucleic acid pools

Rounds 0, 9-spr, 13-rm, 14i, 18b, 19a, and latency-associated protein (LAP) were

15   tested on mink lung epithelial cells for their ability to reverse TGFβ2-mediated inhibition of ^{3}H-thymidine incorporation. The results are that the K_i of the round 9-spr pool was about 711 nM. The K_is of the round 14i, 18b, 19a and 21a pools were about 231 nM, 309 nM, 154 nM and 10 nM, respectively. The K_i of LAP was about 0.5 nM.

From these results it can be concluded that inhibitors of TGFβ2 were enriched in

20   the later rounds of the TGFβ2 SELEX. In addition, there is a continuous correlation between the affinity measured *in vitro* and the inhibitory activity measured *in vivo*:

LAP < round 19A < round 14i < round 13-rm < round 8-spr < round 0.

Sequencing of nucleic acid ligand clones isolated from filter SELEX

Based on several criteria (pool K_d, filter-binding background, bound to noise

25   background, inhibitory activity in cell assay, and absence of aberrant products during the RT-PCR steps of SELEX) round 21a was subcloned for sequence analysis. Forty eight clones were sequenced from round 21a. Two unique sequences represented by clones 21a-4 (SEQ ID NO:86) and 21a-21 (SEQ ID NO:87) (the first number refers to the SELEX round a clone was initially isolated from and the second number is a clone number) were

30   identified (**Table 8**). Several clones were minor variants (1–6 bases different) of clones 21a-4 and 21a-21. One hundred more clones were screened by PCR using primers specific

for clones 21a-4 and 21a-21. Of these, 90 were clone 21a-21-like, 9 were clone 21a-4-like, and 1 was a third unique sequence (21a-48), which was shown to be a nitrocellulose filter-binding sequence. In conclusion, round 21a consists almost entirely of two sequences and variations of those sequences. This was not surprising because round 21a was the second

5   to last round and the bulk affinity of the nucleic acid pools had not improved much from round 19a to 21a.

Since the sequence diversity of round 21a was restricted, 3 other rounds (14i, 16a and 18a) were also sequenced. Only one more novel sequence (14i-1 (SEQ ID NO:72) and variants) was isolated. Two filter-binding sequences were also isolated (16a-1 and a

10   variant of 21a-48). Therefore, as with rounds 8-spr, 13-rm, and 21a, these 3 rounds also did not contain diverse TGFβ2-binding nucleic acid ligands.

The sequences of 14i-1, 21a-4, and 21a-21 are shown in **Table 8.** The affinity of the sequences for human TGFβ2 is about 10 nM, 3 nM and 1 nM respectively. Therefore, these 3 sequences are ligands that bind human TGFβ2 with high affinity.

15   The ligands were tested for inhibitory bioactivity. The K_i of 14i-1, 21a-4, and 21a-21 are about 200 nM, 30 nM and 10 nM respectively. Thus these ligands are also inhibitory ligands. As for the pools the binding affinity correlates well with the inhibitory activity. This is not surprising since it is likely the TGFβ2 ligands bind near the heparin binding site which is very close to the TGFβ receptor binding region. The inhibitory

20   activity of ligand 21a-21 was also compared to that of antibodies.

Clones were isolated and sequenced from six rounds (8-spr, 13-rm, 14i, 16a, 18a, and 21a). The number of each type of sequence is summarized in **Table 9.** Out of 264 clones analyzed by sequencing and 100 clones analyzed by a PCR-based analysis using ligand-specific primers (**Table 10**), only 3 different TGFβ2 ligand sequences (and minor

25   variants) were obtained. Fifteen sequences were filter binding sequences and 36 were nucleic acids that do not bind well to filters or TGFβ2. The degree of restriction in sequence diversity observed in this SELEX is very unusual. Generally one can isolate dozens of different nucleic acid ligands and usually it is possible to find high affinity rounds were one ligand represents <10% of the population.

30   Since sequencing and screening of 6 rounds of SELEX that are as much as 13 rounds apart did not result in a diverse set of sequences the properties of the pools were

investigated further to determine where the sequence diversity was restricted. Selected nucleic acid pools were sequenced and semi-quantitative RT-PCR on nucleic acid pools using ligand-specific primers was done. The results are shown in (**Table 10**). Taken together with the sequencing results, it appears that a restriction in sequence diversity

5   during the SELEX process may have occurred near rounds 6-spr or 7-spr.

Clone 14i-1 is first detectable in round 6-spr, becomes most frequent near round 14i, and decreases in frequency in later rounds. Clone 21a-4 is first detectable by sequencing in round 14i, is most abundant in round 16a, and decreases in frequency by round 21a. However 21a-4 may exist in prior rounds. (RT-PCR analysis of pools using a

10   primer specific for clone 21a-4 was not done.) Clone 21a-21 was rare in round 14i (<1/104 clones by sequencing; estimate <1/200-500 clones by RT-PCR), became more frequent in round 16a, and composes most of round 18b and 21a.

It appears the surface plasmon resonance biosensor SELEX resulted in a high degree of diversity restriction, which has been observed before using this technology. The

15   reason why various later rounds would consist of virtually one sequence is not clear. Perhaps only a very small number of sequences bind TGFβ2 under the selection conditions used. Perhaps a change in selection conditions such as the inclusion of competitors at round 9 or the switch from protein-excess binding reactions to nucleic acid-excess binding reactions at round 16 resulted in the emergence of clone 21a-21 as the predominant clone

20   by round 21a. It seems as though the selection pressures were significant because the predominant ligand in a pool changed in as few as 2 rounds.

The pattern of changes in the population of nucleic acid ligands can be explained by analogy to the theory of natural selection. In an early SELEX round, a variety of sequences will exist. Strong selective pressure may narrow the sequence variation

25   considerably, to the point that a single sequence is predominant. However rare ligands still exist that can be selected in future rounds or during significant changes in selective pressure. This is true in any SELEX experiment, but the TGFβ2 SELEX experiment described here may be an extreme example. In spite of the restriction on sequence diversity, better binding ligands could eventually be isolated. Note that ligand 21a-21 was

30   first identified by sequencing in round 16a. Thus rare, high affinity nucleic acid ligands may exist even in round 22 that would only become predominant under the correct

selection conditions. One approach for isolating such rare sequences might be to specifically deplete late rounds of SELEX of known sequences (e.g., by hybrid selection, restriction enzyme digestion of PCR products, site-directed RNase H cleavage of nucleic acid), an approach that this TGFβ SELEX is well suited for since essentially only 5

5   different sequences (3 ligands and 2 filter binding sequences) were present in later rounds. Isolating a sequence that is present in <1/1000 clones might be easy using depletion methods, but would be tedious using sequencing or PCR screening methods.

These results raise questions about when a SELEX is done and how to judge whether it is done. In this SELEX, standard criteria for judging when a SELEX is done

10   such as K_d improvement, and sequencing of clones or bulk nucleic acid pools may not be good criteria for judging if the SELEX had proceeded as far as it could. Often there are technical limitations (background, reaction volumes, loss of low amounts of protein to large surfaces) that determine when a SELEX must be terminated and these are artificially limiting. Perhaps a "depletion SELEX" round should be done at the end of every SELEX

15   to attempt enrichment of ligands that would be difficult to isolate by currently used methods.

Specificity of human TGFβ2 ligands

For nucleic acid ligands to be most useful in the applications claimed herein they should be highly specific for a particular subtype of TGFβ. The specificity of human

20   TGFβ2 ligands was investigated by in several ways as discussed below.

The specificity of TGFβ2 ligands was examined using the cell culture bioactivity assay where the specificity of the TGFβ2 (described here) and TGFβ1 (see U.S. Patent Application Serial No. 09/046,247, filed March 23, 1998, entitled "High Affinity TGFβ Nucleic Acid Ligands and Inhibitors," which is incorporated herein by reference in

25   its entirety) aptamers was compared to the specificity of antibodies. Two types of antibodies were used namely, monoclonal antibodies and immunopurified polyclonal antibodies. It was found (**Figure 6**) that the TGFβ2 ligand NX22283 (SEQ ID NO:114) inhibited TGFβ2 protein bioactivity (K_i=10 nM), but not TGFβ1 (K_i>1000 nM) or TGFβ3 bioactivity (K_i>1000 nM). The TGFβ2 ligand NX22283 inhibits the TGFβ2

30   bioactivity with a potency equivalent to that of a monoclonal antibody while the most

40

potent inhibitor of TGFβ2 bioactivity in this experiment was an *affinity-purified* polyclonal antibody.

The specificity of a TGFβ2 ligand for TGFβ2 compared to TGFβ3 was also analyzed in nucleic acid binding assays. The affinity of round 0 40N7 nucleic acid or the

5   full-length TGFβ2 ligand 21a-21 to human TGFβ2 protein was >10 μM or 1 nM, respectively. The affinity of round 0 nucleic acid or ligand 21a-21 to human TGFβ3 protein was >10 μM or >30 μM, respectively. Therefore, the TGFβ2 ligand does not bind significantly to TGFβ3.

It was found that the TGFβ1 ligand 40-03 (1 (see U.S. Patent Application Serial

10   No. 09/046,247, filed March 23, 1998, entitled "High Affinity TGFβ Nucleic Acid Ligands and Inhibitors") bound to TGFβ3 although 1000-fold worse. These results indicate there may be one or more amino acids in common between TGFβ1 and TGFβ3 that are not found in TGFβ2 so that a TGFβ1 ligand can bind TGFβ1 and TGFβ3, but not TGFβ2 and so that the TGFβ2 ligand 21a-21 binds TGFβ2 but not TGFβ1 or TGFβ3.

15   Indeed, as shown in **Table 12**, there are 19 amino acids out of 122 that are found in TGFβ2, but not in TGFβ1 or TGFβ3. Three of these differences (Lys-25, Arg-26, and Lys-94 in TGFβ2) are within a putative heparin binding region and may be important for determining the binding specificity of TGFβ ligands.

Truncation of nucleic acid ligands

20   It is desirable to obtain the smallest "truncate" of a full length nucleic acid ligand so that it can be synthesized efficiently at the least cost. The goal of this study was to obtain ligands that are less than half their original length (<35 bases), yet retain about the same affinity as the full length ligand. Several approaches were used to identify truncates of the three TGFβ2 ligands.

25   RNase H and hybrid 2'-OCH$_3$ RNA/DNA oligonucleotides (**5'N7 cleave** (SEQ ID NO:19), **3'N7 cleave** (SEQ ID NO:20), **Table 1**) were used to remove the 5' and 3' fixed sequences from 2'-F pyrimidine, 2'-OH purine nucleic acid ligands as described in U.S. Patent Application Serial No. 09/275,850, filed March 24, 1999, entitled "Truncation SELEX Method," which is hereby incorporated by reference in its entirety.

30   Second, the "boundaries" of the ligands were identified using a previously described method (Fitzwater and Polisky (1996) Meth in Enzymol 267:275-301).

41

Boundaries define the 5' and 3' ends of the smallest truncate. However boundary

determination does not identify internal deletions that can be made. Also because of the

nature of the boundary determination method, if a boundary falls within a run of

pyrimidines or is too close to either end, then which nucleotide is the boundary must be

5   determined by other methods (e.g., generation of ligands beginning or ending with each

candidate boundary position followed by analysis of their binding to TGFβ2).

A third method used relied on plausible structural motifs to define hypothetical

sequence boundaries. Synthetic oligonucleotides corresponding to these boundaries were

synthesized and were tested for binding to TGFβ2.

10   A fourth approach for identifying TGFβ2 ligand truncates was to look at the

location of sequence variations in each ligand. In ligands 21a-4 and 21a-21 the changes

that occurred in sequence variants were distributed randomly throughout their sequences.

However in ligand 14i-1, the sequence changes in variants were highly localized. This

implied that the variable region of ligand 14i-1 could tolerate changes without affecting

15   binding and that the whole variable region may be dispensable.

A fifth approach was to make internal deletions based on predicted structures.

Portions of putative bulges, loops, or base pair(s) within predicted stems can be deleted.

The success of this method depends critically on how close the structural model is to the

real structure. For 21a-21 the most stable structure was found to be incorrect. Only when

20   a structure closer to the real structure was identified (by using the biased SELEX method)

could internal deletions of 21a-21 successfully be made.

Truncation of ligand 14i-1

Using the RNase H truncation method it was determined that ligand 14i-1 requires

the 5,' but not the 3' fixed sequence (**Table 13**). Consistent with this result, when both the

25   5' and 3' fixed sequences were removed, ligand 14i-1 did not bind TGFβ2.

Conventional boundary experiments defined the 3' end of ligand 14i-1 (**Figure 7**)

to be within positions 39-45. In the same experiment we failed to observe a clear

boundary at the 5' end of this ligand. Of the 60 sequence variants of ligand 14i-1, 54 have

nucleotide changes that occur within the last 16 bases at the 3' end of the selected sequence

30   region. Most of the variants have single base changes, but a few have as many as 6 bases

changed. Such changes may or may not affect binding. If they affect binding then that

42

region is important for binding. More likely, since there are so many changes in so many clones within that region, those bases are probably not important. It was surmised they may be able to be deleted, possibly along with the adjacent 3' fixed region, without affecting binding. This idea was confirmed by the following two experiments:

5   1) The binding of 8 sequences that varied within the 16 base region and had different sequence changes was tested. They all bound as well to TGFβ2 as the 14i-1 ligand.
2) A 38 base long truncate of 14i-1 (14i-1t5-41 (SEQ ID NO:131); **Table 13**) that lacked the 3' fixed region and the 16 base variable region bound to TGFβ2 as well as the full length (71 base long) ligand.

10   Four sequences that removed additional bases from the ends of 14i-1 beyond those removed in 14i-1t5-41 were made [(14i-1t5-38 (SEQ ID NO:132), 14i-1t5-35 (SEQ ID NO:133), 14i-1(ML-87) (SEQ ID NO:135), and 14i-1(ML-89) (SEQ ID NO:136)]. Also one internal deletion of 14i-1t5-41 was made [14i-1(ML-86) (SEQ ID NO:134)]. None of these bound to TGFβ2 (**Table 13**). Taken as a whole these experiments showed that the

15   boundaries of ligand 14i-1 fall within positions 5-7 at the 5' end and 39-41 at the 3' end. All these results defined a truncate for ligand 14i-1 that is 38 bases long (**Table 13**).
Truncation of ligand 21a-4 (SEQ ID NO:86)

Using the RNase H truncation method it was determined that ligand 21a-4 requires the 5', but not the 3' fixed sequence (**Table 14**). When both the 5' and 3' fixed sequences

20   were removed ligand 21a-4 did not bind TGFβ2.

The boundaries of ligand 21a-4 (**Figure 7**) are at positions U11 in the 5' fixed region and within positions 52-56 on the 3' end, defining a truncate that is between 42-46 bases long (**Table 8**). This is consistent with RNase H truncation results which show 21a-4 requires the 5' end, but not the 3' end to bind TGFβ2.

25   By examining hypothetical structures, the boundaries for 21a-4 were predicted to occur at position G12 at the 5' end and position C48 at the 3' end. These positions agree well with the region defined by the boundary method. A 37 base long truncate of 21a-4 (excluding sequences required to initiate transcription), beginning at position 12 and ending at position 48 [(21a-4(ML-110) (SEQ ID NO:144); **Table 14**)], bound as well to

30   TGFβ2 as the full length 21a-4 ligand.

43

One sequence that removes 4 additional bases from the 3 end of 21a-4(ML110) was made that is called 21a-4(ML-111) (SEQ ID NO:145). The binding of 21a-4(ML-111) was reduced 30-fold compared to 21a-4(ML-110). Also three internal deletions of 21a-4(ML-110) were made [21a-4(ML-92 (SEQ ID NO:141), ML-108 (SEQ ID NO:142)

5   and ML-109) (SEQ ID NO:143)]. None of these bound well to TGFβ2 (Table 14). A sequence [21a-4(ML-91) (SEQ ID NO:140)] that added 2 bases to the 3' end of 21a-4(ML111) did not have any improved binding compared to 21a-4(ML-110). Thus, the smallest truncate of ligand 21a-4 identified, that retains binding, is 42 bases long.

Truncation of ligand 21a-21 (SEQ ID NO:72)

10   Using the RNase H truncation method it was concluded that ligand 21a-21 requires the 3', but not the 5' fixed sequence (Table 11). However, when both the 5' and 3' fixed sequences were removed, ligand 21a-21 bound TGFβ2. This seems paradoxical since removal of the 3' end alone eliminates binding. However, the data can be interpreted to mean that the 3' deletion folds in a structure that does not bind to TGFβ2, while the

15   truncate that lacks both ends does not fold into a dead end structure. Indeed Mfold structure predictions indicate this may be the case.

          The boundaries of ligand 21a-21 (**Figure 7**) are at position G21 on the 5' end and within positions 50-55 on the 3' end, defining a truncate that is 30-35 bases long. The results are consistent with RNAse H truncation data which shows that 21a-21 requires

20   neither the 5' nor the 3' end to bind TGFβ2. The truncate identified by boundaries falls completely within that defined by RNase H truncation.

          Synthetic sequences based on putative structures were also tested as summarized in **Table 11**. Results from these experiments are in agreement with the RNAse H and conventional boundary experiments.

25   Several additional end truncates and internal deletions of 21a-21(ML-95) were made (**Table 11**). The 9 end truncates included 21a-21(ML-96), 21a-21(ML-97), 21a-21(ML-103) 21a-21(ML-104) 21a-21(ML-105), NX22286, NX22301, NX22302 and NX22303. Of these only NX22301, which removes one base at the 5' end, binds as well as 21a-21(ML-95). Internal deletions included 21a-21(ML-99), 21a-21(ML-101), 21a-

30   21(ML-102), 21a-21(ML-114), 21a-21(ML-115), 21a-21(ML-116), 21a-21(ML-118), 21a-21(ML-120), 21a-21(ML-122), 21a-21(ML-128), 21a-21(ML-132), 21a-21(ML-134), 21a-

44

21(ML-136) and 21a-21(ML-138). Of these 14 internal deletions, only 21a-21(ML-130)
bound about as well as 21a-21(ML-95).

Three sequences [21a-21(ML-94), NX22283 and NX22285), were made that are
longer than 21a-21(ML-95). Of these only NX22285 may have bound (marginally) better

5   than 21a-21(ML-95). Thus, the shortest ligand identified that binds TGFβ2 is the 34-mer
NX22284.

NX22283 and NX22284, which are synthetic analogs of the transcribed ligands
21a-21(ML-94) and 21a-21(ML-95), respectively, bound with identical affinity to TGFβ2
(**Table 11**). The synthetic nucleic acids also have the same inhibitory bioactivity as their

10   transcribed analogs; on the other hand, the short, 30-base long NX22286 and its
transcribed analog 21a-21 (ML-96) do not bind TGFβ2 and they do not inhibit TGFβ2
bioactivity. Therefore synthetic nucleic acids have the same properties as their transcribed
counterparts.

To summarize, truncated 14i-1, 21a-4, and 21a-21 ligands were identified that bind

15   TGFβ2 as well as the full length ligands and are 38, 37 (excluding 5 bases added to
improve transcription yield), and 32 bases long, respectively. Twenty four sequences were
made in an attempt to shorten the NX22284 truncated ligand (8 single base deletions and
16 multiple base deletions). Only 2 of them, (21a-21(ML-130) and NX22301, bind to
TGFβ2. Therefore, it appears that the sequence and spacing of structural elements in

20   NX22284 must be maintained for binding to occur.

Competition between ligands for binding TGFβ2

Examination of the ability of different ligands to compete with each other for
binding to a protein can indicate whether the ligands bind to a similar (or overlapping) or
distinctly different regions on the protein.

25   The ability of NX22283 (SEQ ID NO:114), a truncate of ligand 21a-21 (SEQ ID
NO:87) (**Table 11**), to compete with 4 ligands (14i-1 (SEQ ID NO:72), 21a-4 (SEQ ID
NO:86), 21a-21, and NX22283) was tested. The results were that NX22283 competed
best with itself, then with 21a-21, 21a-4, and 14i-1, in decreasing effectiveness. Thus, the
ability of NX22283 to compete correlates with how related its sequence is to the sequence

30   of the test ligand. NX22283 is most closely related to itself and competes best with itself.
NX22283 is a truncate of 21a-21, and competes with 21a-21 second best. The sequence of

45

21a-4 may be distantly related to 21a-21 and NX22283 competes with 21a-4 third best.
The sequence of ligand 14i-1 is not related to NX22283, and NX22283 is least capable of
competing with 14i-1.

The concentration range of NX22283 required to inhibit 50 % of the binding of the
5   other ligands was 10-fold. Since these differences in the amount of NX22283 it took to
compete off the other ligands can be attributed to differences in their affinity there is
probably only one type of binding region for these ligands on TGFβ2. However, there
may be one or more similar sites per homodimer of TGFβ2. If there were two distinct
types of nucleic acid binding sites on TGFβ2 (as is the case for the HIV-1 gag protein;
10   Lochrie *et al.* (1997) Nuc. Acids Res. 25:2902-2910) it should take >1000 times as much
competitor (i.e., the difference between the K_d of round 0 nucleic acid and the K_d of
NX22283) to compete off a ligand binding at a second distinct site, because presumably a
ligand that has high affinity at one site would have low affinity for a distinct site. This
was not observed.

15   **Off-rate of NX22283**

The half-life for NX22283-TGFβ2 complex was measured in 2 experiments to be
0.5 or 3 minutes. Almost all of the ligand dissociated from TGFβ2 in 60-75 minutes.
Although these times may seem short, they are typical of *in vitro* off-rate measurements
for nucleic acid ligands that have been isolated by filter partitioning SELEX.

20

Example 3. Nucleic acid ligands isolated by the SELEX method using a biased round 0
library

A biased SELEX is one in which the sequences in a nucleic acid pool are altered to
bias the result toward a certain outcome. The primary goals of a "biased" SELEX are to
25   obtain ligands that have a higher affinity and to determine what the putative secondary
structure of a ligand may be. The starting, round 0 nucleic acid library (called 34N7.21a-
21) used for the TGFβ2 biased SELEX had the same 5' and 3' fixed regions (5'N7 and
3'N7) as the prior TGFβ2 SELEX (**Table 1**). It was made as a 2'-F pyrimidine, 2'-OH
purine nucleic acid. However, as described in Example 1, the random region was 34 bases
30   long. Within the randomized region 62.5% of the nucleotides at each position correspond
to the NX22284 (SEQ ID NO:115) sequence. The remaining 37.5% correspond to the

other three nucleotides. Thus each position is mutagenized and the sequence of the pool is biased toward the NX22284 sequence. Selection for ligands that bind to TGFβ2 using such a pool should allow variants of NX22284 to be isolated, some of which may not have been present in the original 30N7 round O pool.

5   The bulk K_d of the round 0 34N7.21a-21 pool was about 870 nM (**Table 15**) using protein-excess binding conditions. This is at least 10-fold better than for the unbiased round 0 40N7 pool, as would be expected. This round 0 nucleic acid pool also bound under nucleic acid-excess conditions in small scale SELEX type reactions, although poorer than in protein-excess reactions, as would be expected. The progress of the biased SELEX

10   is shown in **Table 15**.

          The conditions used in the biased SELEX and the results are shown in **Table 15**. A total of 9 rounds were done. Attempts were made to obtain higher affinity ligands by using competitors, starting at round 4. Both yeast tRNA (low affinity) and NX22284 (high affinity) were used as competitors. Both are nonamplifiable during the PCR step of

15   SELEX. The "A" series was done without competitors while the "B" series was done with competitors.

          The binding of the nucleic acid pools to TGFβ2 was measured for rounds 0 to 8 (**Table 15**) and found to improve from ~870 nM for the round 0 nucleic acid library to ~1 nM for the round 5a nucleic acid pool. Competition seemed to have little consistent effect

20   on affinity improvement in this SELEX experiment. Probably competition should have been initiated with NX22284 at round 1. Peak improvement in the pool affinity plateaued in rounds 5, 6 and 7, and 8. Therefore round 5a , the earliest round with the best affinity, was subcloned and sequenced.

Sequences of TGFβ2 nucleic acid ligands obtained from a biased SELEX.

25   As shown in **Table 16**, 25 unique sequences were obtained. One to nine changes from the starting sequence were found. All of the clones were 34 bases long within the selected sequence, consistent with studies (see "Truncation of ligand 21a-21" above) where it was difficult to delete any internal bases.

          Covariance between pairs of positions was analyzed by eye and by using the

30   consensus structure matrix program (Davis *et al.* (1995) Nucleic Acids Research 23:4471-4479). Covariance was observed between 2 different areas implying the existence of 2

47

stems in the structure. The pattern of covariance suggests the structural model shown in
Figure 8 or a similar variant of that structure (e.g., some base pairing could occur within
the loop). This predicted structure is the third most stable structure predicted by the Mfold
program (Zuker (1989) Science 244:48-52). A curious example of possible covariance is

5   observed at positions 15 and 25 in the loop region. A15 and G25 were observed to covary
to C15 and U25 in 2 clones (#18 and #29). Ligand 21a-4 also has the C/U combination at
the bottom of its putative loop.

Of 34 bases, 11 are "invariant" among these 25 clones (**Table 16**). All of the
invariant positions are predicted to occur in the loop and bulge regions except C34, the last

10   nucleotide. The last base of all 3 truncated TGFβ2 ligands (**Figure 8**) is a C. Removal of
this C results in loss of binding. If invariant positions indicate regions where TGFβ2
binds the NX22284 ligand, then binding may occur primarily in the bulge and stem loop
regions. The stems must be base paired, but can vary in sequence implying that the
structure of the stems may be more important than their sequence. The stem may be a

15   structure used to present the bulge, loop and C34 nucleotides in the proper orientation to
bind TGFβ2.

Clone 5a-11 from the biased SELEX is similar to clone 21-4 from the primary
SELEX, particularly at positions that are invariant in clones from the biased SELEX, thus
reinforcing the new structural model and the importance of the invariant positions. It has

20   not been possible to fit ligand 14i-1 into a similar structure. Perhaps it represents a second
sequence motif capable of binding TGFβ2.

Binding of nucleic acid ligands isolated from the biased SELEX

The binding of clones from the biased SELEX was compared to the binding of full
length ligand 21a-21. The majority of the clones bound as well to TGFβ2 as 21a-21

25   (**Table 16**). One clone (#20 (SEQ ID NO:160)) bound about 6-fold worse and one clone
(#13 (SEQ ID NO:154)) bound about 5-fold better than full length ligand 21a-21. The
average K_d of the clones (weighting clones found more than once) is 1.2 nM, which agrees
with the round 5a pool K_d of ~1 nM. Thus, the ligands that were isolated in this manner
were not vastly different in affinity from the starting sequence.

30   One would expect there to be an optimal number of changes that results in higher
affinity ligands. Clones with only a few changes might be expected to bind about the

48

same as the starting sequence, clones with a threshold number of changes may bind better, and clones with too many changes may bind worse. Indeed there may be a correlation between the number of changes and the affinity. Clones with 1 to 4 changes tend to bind the same or worse than ligand 21a-21. Clones with 5-8 changes tend to bind better than

5   ligand 21a-21. The worst binder (#20) was the one with the most changes (9). The ligand that bound to TGFβ2 the best (clone #13) had 7 changes relative to the starting sequence.

When the clones that bound better and those that bound worse are aligned (**Table 17**) it appears that an A at position 5 may be important for higher affinity binding since the ligands that bind to TGFβ2 best all have an A at position 5 and all clones with an A at

10   position 5 bind at least as well as 21a-21. In contrast clones with a U, C or G at position 5 tend to bind worse than 21a-21. With regard to the pattern of base pair changes in the putative stems there is no single change that correlates with better binding. In addition, the better binders do not consistently have GC-rich stems. However the pattern of changes in the stems of the poor binders does not overlap with that seen in the stems of the better

15   binders. Thus, various stem sequences may result in better binding for subtle reasons.

A point mutant that eliminated binding of the full length 21-21 transcript (21a-21(ML-107); **Table 11**) changes U at position 6 to G. A G was found at position 6 in three clones from the biased SELEX (#4, 9 and 35), one of which (clone #4) has only one other base change while the others had additional changes. All three clones from the

20   biased SELEX that have a G at position 6 bind TGFβ2. Thus it would seem that the U6G change alone eliminates binding, but this binding defect can be reversed when combined with other sequence changes.

To summarize, some changes (such as A at position 5) may act independently and be able to confer better binding alone, while others changes (e.g., at position 6 and in the

25   stems) may influence binding in a more unpredictable way that depends on what other changes are also present.

Presumably sequences that lack an "invariant" nucleotide would not bind to TGFβ2. Some of the invariant bases have been deleted and others have been changed (**Table 11**). None of these 10 altered sequences [21a-4(ML-111); 21a-21(ML-96, 97, 101,

30   102, 103, 104, 105, 120, NX22286] bind to TGFβ2.

Example 4. <u>Substitutions of 2'-OH purines with 2'-OCH₃ purines in NX22284</u>

Substitutions of 2'-OH purines with 2'-OCH₃ purines sometimes results in nucleic acid ligands that have a longer half life in serum and in animals. Since the nucleic acid ligands described here are ultimately intended for use as diagnostics, therapeutics, imaging, or histochemical reagents the maximum number of 2'-OH purines that could be substituted with 2'-OCH₃ purines in ligand NX22284 was determined. NX22284 is a 34-mer truncate of the 70 base long 21a-21 TGFβ2 ligand (**Table 18**). NX22284 has 17 2'-OH purines and binds about 2-fold worse than ligand 21a-21.

Initially an all 2'-OCH₃ purine substituted sequence was synthesized (NX22304). Another sequence has all 2'-OH purines substituted with 2'-OCH₃ purines except six purines at its 5' end. Neither bound to TGFβ2 or had measurable bioactivity (**Table 18**).

Therefore a set of sequences was synthesized (NX22356-NX22360; **Table 18**) such that groups of 3 or 4 2'-OH purines were substituted with 2'-OCH₃ purines. The binding of NX22357 was reduced about 2-fold and the bioactivity was reduced 10-fold. The binding and bioactivity of NX22356, NX22258 and NX22360 were unaffected. In contrast the binding of NX22359 was reduced over 100-fold and its bioactivity was reduced over 30-fold. Therefore, the sequence of NX22359 was "deconvoluted" one base at a time in order to determine which individual purines in NX22359 cannot be 2'-OCH₃ purines. NX22374, NX22375 and NX22376 are deconvolutions of NX22359. All three of these sequences had greatly reduced binding and bioactivity. This suggests that G20, A22 and A24 cannot be 2'-OCH₃ purines.

NX22377 was designed to determine if a sequence with an intermediate number of 2'-OCH₃ purines could bind TGFβ2 and retain bioactivity. NX22377 has 10 2'-OCH₃ purines out of 17 (representing the 2'-OCH₃ purines in NX22356, NX22357 and NX22360). The binding and bioactivity of NX22377 are identical to NX22284.

NX22417 was designed to test the possibility that G20, A22 and A24 must be 2'-OH purines in order to retain binding and bioactivity. In NX22417 G20, A22, and A24 are 2'-OH purines while the other 14 purines are 2'-OCH₃. NX22417 binds to TGFβ2 as well as NX22284, but its bioactivity is reduced about 10 fold. Since substitution of G20 (NX22374) or A24 (NX22376) alone had a less severe effect than substitution of A22 (NX22375), nucleic acids were synthesized that had all 2'-OCH₃ purines except position A22 (see NX22384) or G20 and A22 (see

50

NX22383). NX22383 and NX22384 did not bind or inhibit TGFβ2, again suggesting that at least 3 purines at positions 20, 22, and 24 must be 2'OH to retain binding and bioactivity.

NX22384 was analyzed by mass spectroscopy to ensure its lack of binding and inhibitory activity was not due to incomplete deprotection or an incorrect sequence. The results are that NX22383 may be 0.5- 0.9 daltons more than the predicted molecular weight and therefore is very likely to be what it should be.

Since NX22357 bound to TGFβ2 slightly worse than NX22284, but had a 10-fold reduced bioactivity, it was possible that one or more of the three 2'-OCH$_3$ purines in NX22357 (G5, A8 or A11) may also be required for bioactivity. This notion was tested by synthesizing NX22420 and NX22421. NX22421 has all three of these bases (G5, A8, and A11) as 2'-OH purines (along with G20, A22 and A24, which require 2'-OH groups). NX22420 has A8 (along with G20, A22 and A24) as 2'-OH purines. NX22421 has G5, A8 and A11 (along with G20, A22 and A24) as 2'-OH purines. A8 was retained as a 2'-OH purine in both NX22420 and NX22421 because it was invariant among the clones from the biased SELEX and therefore it was inferred that A8 might be less tolerant to change at the 2' ribose position (as was the case for G20, A22 and A24). Indeed both NX22420 and NX22421 had approximately the same binding and inhibitory activity as NX22284. In summary, the NX22284 sequence can retain maximal binding and inhibitory activity when four purines (A8, G20, A22 and A24) are 2'-OH and the other purines are 2'-OCH$_3$. Note that all four of these positions were invariant among the clones isolated using the biased SELEX method.

While studies were being done on substituting the 2'-OH purines of NX22284, two shorter versions of NX22284 (21a-21[ML-130] and 21a-21[ML-134]; **Table 11**) were discovered that bound well to TGFβ2 as transcripts. The 2'-OCH$_3$ purine substitution pattern of NX22420 was transferred to these sequences. NX22426 is the 2'-OCH$_3$ purine analog of 21a-21(ML-134) and NX22427 is the 2'-OCH$_3$ purine analog of 21a-21(ML-130). NX22426 bound well to TGFβ2, but had 25-fold reduced bioactivity. NX22427 may have slightly better binding and inhibitory activity than NX22284.

In summary, the human TGFβ2 ligand isolated by using combined spot, spr, and filter SELEX methods which have the best combination of affinity, short length, and

51

inhibitory activity is NX22427, a 32-mer with 12 2'-OCH$_3$ purines out of a total of 16

purines.

Substitutions of 2'-OH purines with 2'-OCH$_3$ purines in NX22385

Some of the ligands that were isolated using the biased SELEX method (e.g., clone

5   13) bound better to TGFβ2.

To compare the properties of a truncated clone 13 to truncated 21a-21, NX22385 was

synthesized. NX22385 (**Table 19**) is a 34 base long, 2'-F pyrimidine, 2'-OH purine version of

biased SELEX clone #13. It binds about 2.5-fold better than NX22284, the corresponding 34 base

long truncate of 21a-21, but its inhibitory activity is about 4-fold worse.

10   For reasons mentioned in the previous section it was of interest to determine if the

properties of a truncated clone 13 t when synthesized as a 2'-F pyrimidine, 2'-OCH$_3$ purine nucleic

acid. Two 2'-OCH$_3$ purine versions of NX22385 (NX22424 and NX22425; **Table 19**) were

synthesized based on the 2'-OCH$_3$ pattern of NX22420, a truncate of 21a-21. In both nucleic acids

A8, G20, A22 and A24 were retained as 2'-OH purines, as in NX22420. In NX22424, the purines

15   that are unique to clone 13 (A5, A6 and G12) are 2'-OH purines. In NX22425, those purines are

2'-OCH$_3$ purines. Analogs of NX22424 and NX22425 were also synthesized in which A24

(NX22386) or G20 and A24 (NX22387) are 2'-OCH$_3$ purines. NX22386 and NX22387 were

expected to serve as negative controls since 2'-OCH$_3$ G20 or A24 version of NX22284 were

inactive. As expected NX22386 and NX22387 did not bind or inhibit TGFβ2. NX22424 and

20   NX22425 bound to TGFβ2 as well as NX22284, but were reduced >100-fold in bioactivity (

Table 19). Therefore, while other sequences that bind as well as NX22284 were isolated, no

other sequence was identified that have better bioactivity.

Example 5. <u>Pharmacokinetic properties of NX22323</u>

25   NX22323 is a 5'-polyethylene glycol-modified version of NX22284 (see **Table 11;**

Figure 9). The plasma concentrations of NX22323 were measured in rats over a 48 hour

time period and are shown in **Figure 11** with the corresponding pharmacokinetic

parameters in **Tables 20** and **21**. These data demonstrate biphasic clearance of NX22323

from plasma with an initial clearance half life (α T$_{1/2}$) of 1 hour and a terminal clearance

30   half life (β T$_{1/2}$) of 8 hours. The volume of distribution at steady state was approximately

140 mL/kg suggesting only minor distribution of the aptamer with the majority remaining

in plasma and extracellular water. The clearance rate determined by compartmental analysis was 0.40 mL/(min*kg). This value was consistent with other aptamers with similar chemical composition (5'-PEG 40K, 3'-3' dT, 2'F pyrimidine, 2'-OH purine nucleic acid). These data support daily administration of NX22323 for efficacy evaluation.

Example 6. 2' OMe Modification of Lead Truncate Ligand CD70

TGFβ1 nucleic acid ligands are disclosed in U.S. Patent Application Serial No. 09/275,850, filed March 24, 1999, entitled "Truncation SELEX Method," which is incorporated herein by reference in its entirety. A lead aptamer was generated by truncation SELEX by hybridization (see **Table 11**, Family 4, Ligand #70 in U.S. Patent Application Serial No. 09/275,850), herein called CD70. CD70 derivative oligonucleotides were synthesized containing 2' OMe modifications at various positions as summarized in **Table 22**. The results suggest that 13 out of 16 purines can be substituted with their 2'OMe counterparts without any loss of activity. The molecule with the maximum 2'OMe modifications (CD70-m13) is also bioactive (**Table 22**). **Figure 10** shows a putative structure of CD70-m13 (SEQ ID NO:206) and the positions of that require the presence of 2'OH nucleotides. Of interest is the A position at the 3' end of the molecule which according to the proposed structure does not participate in a secondary structure. Deletion of this single stranded A affects somewhat the binding activity of the molecule but it completely eliminates its bioactivity (**Table 22**). The 2'OH bases and the 3' final A are in close proximity in the proposed structure. This suggests a domain of the molecule responsible for target binding. Under these circumstances, it is expected that the loop shown at the top of the proposed structure (**Figure 10**) may not be necessary for binding. This was confirmed by replacing such a loop with a PEG linker and showing that such modified molecules retain binding (**Table 22**). The PEG linker was conjugated to the aptamer as shown in U.S. Patent Application Serial No. 08/991,743, filed December 16, 1997, entitled "Platelet Derived Growth Factor (PDGF) Nucleic Acid Ligand Complexes," which is hereby incorporated by reference in its entirety. The shortest binding aptamer identified from these experiments is CD70-m22 (SEQ ID NO:215), a 34-mer (including the PEG linker).

53

Example 7. Mink Lung Epithelial Cell (MLEC) PAI Luciferase Assay

The MLEC PAI Luciferase Assay was performed in order to test the ability of
TGFβ aptamers to interfere with the biological activity of TGFβ *in vitro*. Mink Lung
Epithelial Cells were transfected with a PAI/Luciferase construct that allows for the direct

5   measurement of PAI promoter upregulation by TGFβ. Both TGFβ1 and TGFβ2 can
upregulate the PAI promoter. Stimulation of PAI/Luciferase expression results in a
quantifiable light emission when the Luciferase substrate Luciferin is present. MLEC-
PAI-Luc cells between P_{6-15} were plated at 3×10^4 cells per well in MEM supplemented
with 10 mM HEPES and 0.2% FBS (MEM-S), and allowed to adhere for 4-5 hours. Serial

10   dilutions (1:3) of inhibitors (antibodies or aptamers) were prepared in MEM-S for
incubation with MLEC. Two columns of wells were maintained for each TGFβ and
untreated control groups. TGFβ(1 or 2) was added to each well, except for the untreated
control cells, to 10 pM or 20 pM and the cells were incubated for 15-16 hours. Following
TGFβ stimulation, MEM-S was replaced with DPBS supplemented with Ca^{2+} and Mg^{2+} at

15   1 mM each. Cells were processed with the Luc-lite kit (Packard Instruments) per
manufacturer's instructions. Relative luciferase activity was determined by
chemiluminescence detection (Top Count, Packard Instruments) after a 10 minute dark
adaptation. Data were generated as CPS (counts per second). The results are set forth in
Figures 12 and **13**. It is clear from these figures that both TGFβ aptamers interfere with

20   the biological activity of their respective cytokines. The apparent Ki in these experiments
is about 200 nM.

54

TABLE 1. Sequences used during SELEX.
(all are shown in a 5' to 3' direction, and separated by a blank every 10 bases)

Sequences involved in SELEX process:

(P0; DNA template for round 0 of spot SELEX)
TCGGGCGAGT CGTCTGNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN
NNNNNNCCGC ATCGTCCTCC C 71 (SEQ ID NO: 1)
A=dA; C=dC; G=dG; T=dT; N+25% each of dA, dC, dG, or dT

(5'N7; primer used in PCR steps of SELEX)
TAATACGACT CACTATAGGG AGGACGATGC GG 32 (SEQ ID NO: 2)
A=dA; C=dC; G=dG; T=dT

(3'N7; primer used in RT and PCR steps of SELEX)
TCGGGCGAGT CGTCTG 16 (SEQ ID NO: 3)
A=dA; C=dC; G=dG; T=dT

(Transcription template for round 0 of spot SELEX)
TAATACGACTCACTATAGGGAGGACGATGCGG-40N-CAGACGACTCGCCCGA
(SEQ ID NO:4)
ATTATGCTGAGTGATATCCCTCCTGCTACGCC-40N-GTCTGCTGAGCGGGCT
(SEQ ID NO: 5)
A=dA; C=dC; G=dG; T=dT; N=25% each of dA, dC, dG, or dT

(R0 40N7; nucleic acid library for round 0 of spot SELEX)
GGGAGGACGA UGCGGNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN
NNNNNCAGAC GACUCGCCCG A 71 (SEQ ID NO: 6)
A=2'-OH A; C=2'-F C; G=2'-OH G; N=25 % each of 2'-OH A, 2'-F C, 2'-OH G,
and 2'-F U; U=2'-F U

(34N7.21a-21 DNA template for round 0 of biased SELEX)
GGGAGGACGA TGCGGNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNC
AGACGACTCG CCCGA 65 (SEQ ID NO: 7)
A=dA; C=dC; G=dG; T=dT, N=62.5 % NX22284 sequence as DNA and 12.5% of
the other 4 nucleotides (dA, dC, dG, or dT) at each position

(Transcription template for round 0 of biased SELEX)
TAATACGACTCACTATAGGGAGGACGATGCGG-34N-CAGACGACTCGCCCGA
(SEQ ID NO: 8)
ATTATGCTGAGTGATATCCCTCCTGCTACGCC-34N-GTCTGCTGAGCGGGCT
(SEQ ID NO: 9)

A=dA; C=dC; G=dG; T=dT, N=62.5 % NX22284 sequence as DNA and 12.5% of the
other 4 nucleotides (dA, dC, dG, or dT) at each position

TABLE 1. (Continued)

(34N7.21a-21 nucleic acid library for round 0, biased SELEX)
GGGAGGACGA UGCGGNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNC
AGACGACUCG CCCGA 65 (SEQ ID NO: 10)
A=2'-OH A; C=2'-F C; G=2'-OH G; N=62.5 % NX22284 sequence and 12.5% of other 4
nucleotides (2'-OH A, 2'-F C, 2'-OH G, or 2'-F U) at each position; U=2'-F U

Sequences used for subcloning, screening, sequencing ligand

(ML-34; used for subcloning)
CGCAGGATCC TAATACGACT CACTATA 27 (SEQ ID NO: 11)
A=dA; C=dC; G=dG; T=dT

(ML-78; used for subcloning)
GGCAGAATTC TCATCTACTT AGTCGGGCGA GTCGTCTG (SEQ ID NO: 12)
A=dA; C=dC; G=dG; T=dT

(RSP1 ; vector-specific primer used to screen transformants for ligand inserts)
AGCGGATAAC AATTTCACAC AGG 23 (SEQ ID NO: 13)
A=dA; C=dC; G=dG; T=dT

(FSP2; vector-specific primer used to screen transformants for ligand inserts)
GTGCTGCAAG GCGATTAAGT TGG 23 (SEQ ID NO: 14)
A=dA; C=dC; G=dG; T=dT

(RSP2; primer for sequencing ligands)
ACTTTATGCT TCCGGCTCG 19 (SEQ ID NO: 15)
A=dA; C=dC; G=dG; T=dT

Sequences used to detect specific ligands
(ligand 14i-1 specific primer; ML85)
GCCAAATGCC GAGAGAACG 19 (SEQ ID NO: 16)
A=dA; C=dC; G=dG; T=dT

(ligand 21a-4 specific primer; ML-79)
GGGGACAAGC GGACTTAG 18 (SEQ ID NO: 17)
A=dA; C=dC; G=dG; T=dT

(ligand 21a-21 specific primer; ML-81)
GGGAGTACAG CTATACAG 18 (SEQ ID NO: 18)
A=dA; C=dC; G=dG; T=dT

TABLE 1. (Continued)

<u>Sequences used for RNAse H cleavage</u>
(5'N7 cleave)
CCGCaugcuc cuccc   15 (SEQ ID NO: 19)
a=2'-OCH$_3$ A; c=2'-OCH$_3$ C; C=dC; g=2'-OCH$_3$ G; G=dG; u=2'-OCH$_3$ U

(3'N7 cleave)
ucgggcgagu cgTCTG 16 (SEQ ID NO: 20)
a=2'-OCH$_3$ A; c=2'-OCH$_3$ C; C=dC; g=2'-OCH$_3$ G; G=dG; u=2'-OCH$_3$ U; T=dT

Table 2. Conditions and results of filter SELEX

Round[a]	[RNA][b] nM	[TGFβ2] nM	RNA[b]/protein	[Competitor]	% Bound	% Background	Bound/Background	Kd (nM)
9b	1 nM	100 nM	0.01	100 μM tRNA	4.2	1.1	4	nd
10b	1 nM	30 nM	0.03	100 μM tRNA	4.3	0.13	33	100
11a	1 nM	30 nM	0.03	100 μM tRNA	1.5	0.2	8	75
12d	0.2 nM	20 nM	0.01	250 μM tRNA	2.2	0.3	7	40
13i	0.4 nM	10 nM	0.04	10 μM tRNA	2.6	0.16	16	30
14i	0.1 nM	10 nM	0.01	10 μM heparin	14.5	0.55	20	75
15c	10 nM	10 nM	1.0	0	8.8	2.2	4	30
16a	55 nM	10 nM	5.5	0	9.6	2.1	5	10
17a	30 nM	3 nM	10	0	1.9	0.17	11	5
18b	15 nM	3 nM	5	0	2.3	0.6	4	5
19a	7 nM	0.1 nM	70	0	0.17	0.05	3	2
20a	0.33 nM	0.03 nM	11	0	0.1	0.04	3	1
21a	0.63 nM	0.03 nM	21	0	0.3	0.1	3	1
22a	0.07 nM	0.01 nM	7	0	0.12	0.09	1	1

[a]Number designates the round of SELEX and letter designates the condition used for that round.
[b]NA, nucleic acid library
Only those rounds that were carried to the next round are shown

Table 3. Conditions and results of Spot SELEX

Rd	Protein (pmoles)	RNA (pmoles)	Washes[1] (μl/min)	Signal/Noise	% Input	Incubation	Pre-adsorb[2]
1	*200	2000	2 (500/10)	4.90	ND[3]	4 hrs, 20°C	No
2	*200	1500	2 (1000/10)	1.80	ND	0.5 hrs, 37°C	5 layers, 0.75hrs
3	*200	1500	2 (1000/10)	5.50	ND	1 hr, 37°C	5 layers, 1 hr
4	200	1000	2 (1000/10)	11.20	0.18	1 hr, 37°C	5 layers, 2.5 hrs
	*67	1000	2 (1000/10)	3.70	0.06	1 hr, 37°C	5 layers, 2.5 hrs
	22	1000	2 (1000/10)	1.58	0.03	1 hr, 37°C	5 layers, 2.5 hrs
5	67	100	2 (1000/20)	26.00	1.30	1 hr, 37°C	10 layers, 0.75hrs
	*22	100	2 (1000/20)	11.00	0.56	1 hr, 37°C	10 layers, 0.75hrs
	7.3	100	2 (1000/20)	2.70	0.10	1 hr, 37°C	10 layers, 0.75hrs
6	22	50	2 (1000/20)	20.70	1.00	1 hr, 37°C	10 layers, 0.75hrs
	*7.3	50	2 (1000/20)	4.00	0.20	1 hr, 37°C	10 layers, 0.75hrs
	2.4	50	2 (1000/20)	1.20	0.06	1 hr, 37°C	10 layers, 0.75hrs
7	22	7	3 (1000/50)	24.00	1.30	1 hr, 37°C	10 layers, 1.5hrs
	*7.3	7	3 (1000/50)	7.50	0.40	1 hr, 37°C	10 layers, 1.5hrs
	2.4	7	3 (1000/50)	1.50	0.07	1 hr, 37°C	10 layers, 1.5hrs
8	*7.3	3	2 (1000/60)	77.00	0.41	0.75 hr, 37°C	10 layers, 1.5hrs
	2.4	3	2 (1000/60)	8.50	0.04	0.75 hr, 37°C	10 layers, 1.5hrs
	0.7	3	2 (1000/60)	1.00	ND	0.75 hr, 37°C	10 layers, 1.5hrs
9	*7.3	1	2 (1000/20)	87.00	0.23	1 hr, 37°C	10 layers, 1.5hrs
	2.4	1	2 (1000/20)	4.00	0.01	1 hr, 37°C	10 layers, 1.5hrs
	0.7	1	2 (1000/20)	2.50	0.006	1 hr, 37°C	10 layers, 1.5hrs
10	7.3	<1 (no tRNA)	2 (1000/20)	13.70	ND	0.5 hr, 37°C	10 layers, 1.5hrs
	7.3	<1 (10^1 tRNA)[4]	2 (1000/20)	10.50	ND	0.5 hr, 37°C	10 layers, 1.5hrs
	7.3	<1 (10^2 tRNA)	2 (1000/20)	5.00	ND	0.5 hr, 37°C	10 layers, 1.5hrs
	7.3	<1 (10^3 tRNA)	2 (1000/20)	1.80	ND	0.5 hr, 37°C	10 layers, 1.5hrs

*pool carried to next round
[1]Number of washes, volumes and duration
[2]Number of filters and duration of incubation during the background counterselection step
[3]ND, not determined
[4]Fold excess tRNA over the aptamer pool

Table 4. Conditions and results surface plasmon resonance biosensor (spr) SELEX.

Progress of BIA SELEX with TGFβ2

Rd	TGFβ2, RU[1]				[RNA], μM[2]	Injections (vol, μL)[3]	Fractions (min each)[4]	Fraction FW[5]	RU after SDS[6]
	FC1	FC2	FC3	FC4					
2	1293	874	294	0	4	4 (40)	3 (5)	3rd & SDS	~100
3	1176	1178	1181	0	15	4 (40)	3 (5)	3rd & SDS	~50-100
4	3010	2037	1767	0	10	6 (40)	3 (5)	3rd & SDS	~80
5	5520	5334	4265	0	5	6 (40)	3 (5)	3rd & SDS	~100-150
6	4075	3143	298	0	5	6 (40)	3 (5)	3rd & SDS	~75-100
7	3773	2616	2364	0	2	6 (40)	3 (5)	3rd & SDS	~330-220
8	2574	1842	1461	0	5	4 (40)	3 (5)	3rd & SDS	~60-105
9	3180	2029	1688	0	3	4 (40)	3 (5)	3rd & SDS	~77-114
10	344	718	1692	0	1	4 (40)	6 (10)	6th & SDS	~50
11	217	675	386	0	5	2 (40)	6 (10)	6th & SDS	~50-62

[1]Amount of TGFβ2 immobilized expressed in resonance units where 1RU corresponds to 1pg of protein per mm^2. The protein is immobilized in an area of 1.2 mm^2
[2]concentration of RNA pools
[3]Number of injections and volume of each injection
[4]Number and length in min (in parentheses) of each fraction
[5]Fractions carried to the next round
[6]Amount of RNA eluted after SDS treatment expressed in response units

FC1, FC2, FC3, and FC4 designate the four flowcells of the BIA chip.

Table 5.   Sequences isolated from round 8 of surface plasmon resonance SELEX.

NAME[a]	SEQ ID NO:	SEQUENCE[b]	BINDING[c]
8.1(1)	21	GGGAGGACGAUGCGG UCCUCAAUG-AUCUU--------UCCUGUUUAUGCUCCC CAGACGACUCGCCCGA	FILTER
8.2(1)	22	GGGAGGACGAUGCGG AAGUAACGUUUAAGUAAAAAUUCGUUCUCUCGGUAUUUGGC CAGACGACUCGCCCGA	TGFβ2
8.3(14)	23	GGGAGGACGAUGCGG AAGUAACGUUGAAGUAAAAAUUCGUUCUCUCGGCAUUUGGC CAGACGACUCGCCCGA	TGFβ2
8.5(1)	24	GGGAGGACGAUGCGG UCCUAACCAUCACAAUCUCCAAUCUUAUAUAUUUCCCGCCC CAGACGACUCGCCCGA	NONE
8.6(1)	25	GGGAGGACGAUGCGG --AAACCAAAAGACCACCACAUCUCCAUACGCUCUGCCC CAGACGACUCGCCCGA	NONE
8.8(1)	26	GGGAGGACGAUGCGG AUAGAUCGGUCCGAUAAGUCUUUACCUGGCCCC CAGACGACUCGCCCGA	NONE
8.9(4)	27	GGGAGGACGAUGCGG AAGUAACGUUGAAGUAAAAAUUCGUUCUCUCGGUAUUUGGC CAGACGACUCGCCCGA	TGFβ2
8.11(1)	28	GGGAGGACGAUGCGG ACGAUCCUUUCCUUAAACAUUUCAUUUCUCCUGUGCCC CAGACGACUCGCCCGG	FILTER
8.12(1)	29	GGGAGGACGAUGCGG UCCAUCAACAAUCUUAUCAUUAUGUUUUUCCUUCCCGCCC CAGACGACUCGCCCGA	NONE
8.13(1)	30	GGGAGGACGAUGCGG UCCUCUGAGCCGAUCUUCACUCAUCUUUUCUCUGCCC CAGACGACUCGCCCGA	FILTER
8.15(2)	31	GGGAGGACGAUGCGG UUCCUCAAUUCUUCCAUUCCAUUCAUAAUGUUUCCCUUGCCC CAGACGACUCGCCCGA	FILTER
8.18(1)	32	GGGAGGACGAUGCGG UCUACCCUUAGCAGUAUUGUUUUCCAUCGUUGUUUGCCC CAGACGACUCGCCCGG	NONE
8.20(1)	33	GGGAGGACGAUGCGG UCUCAACGAAGAACACGUUGGAUACUGUUUUCCCGCCC CAGACGACUCGCCCGA	NONE
8.21(1)	34	GGGAGGACGAUGCGG UUCAGUUCCUUCAGUUUCUAAUUCUGUUCCACCUGUCCC CAGACGACUCGCCCGA	FILTER
8.22(1)	35	GGGAGGACGAUGCGG -------AGCGGAUUAAUUAGUCAUGUCCUUCGACUUCUUGUCCC CAGACGACUCGCCCGA	
8.23(1)	36	GGGAGGACGAUGCGG AGACAUCUUGUCUCGAUUAGUAGCUCAUGUUCCUUACCUGCCC CAGACGACUCGCCCGA	NONE
8.24(1)	37	GGGAGGACGAUGCGG --UCCUCUAGCAAGCAGCAUUGUUAUAACGGUAUGCGUCCC CAGACGACUCGCCCGA	
8.25(1)	38	GGGAGGACGAUGCGG UGCACAGUGAUGGAUGACAUUGUAUAGUCAUAGUUUACUCCCGCCC CAGACGACUCGCCCGA	
8.26(1)	39	GGGAGGACGAUGCGG -ACCUAUCUUCUCCAAGUCAUAGUCAUCCGCUAAUCUCCGCCC CAGACGACUCGCCCGA	FILTER
8.28(1)	40	GGGAGGACGAUGCGG AUGAGACCUAAUCAUCGAUCCGCUAAAACCUCACCC CAGACGACUCGCCCGA	NONE
8.29(1)	41	GGGAGGACGAUGCGG UCCUCAGACAAAUCUUCUUGAAUCUUUAACUGCCC CAGACGACUCGCCCGA	FILTER
8.31(1)	42	GGGAGGACGAUGCGG -ACCGAUUCUUCCAAUCUCUUCGCUAUUUUUCUGCCC CAGACGACUCGCCCGA	FILTER
8.33(1)	43	GGGAGGACGAUGCGG UCCUCUGAGCCAAUCUUCGCUACUCUUUUUCUCUGCCC CAGACGACUCGCCCGA	FILTER
8.34(1)	44	GGGAGGACGAUGCGG AUUCUUUCUCCAACGCUUUUCAACAUUAAAUCAUCUUCUCCGCCC CAGACGACUCGCCCGA	FILTER
8.35(1)	45	GGGAGGACGAUGCGG AUCCUAUCUUCGAAUAAUCAUUAAAUCAUCUUCUCCGCCC CAGACGACUCGCCCGA	NONE
8.36(1)	46	GGGAGGACGAUGCGG UUCAAUCAUCUCACUCU-CAUUUCCUUUUCCUACUCCC CAGACGACUCGCCCGA	FILTER
8.38(1)	47	GGGAGGACGAUGCGG CGAUAGAAUCUAGUCGUUCUAGAUGAUCUGGUACGUGCCC CAGACGACUCGCCCGA	FILTER

Table 5. (Continued)

Name				FILTER
8.39(1) 48	GGGAGGACGAUGCGG	UAGUAAUCCUUGUCUUCCAUUUCUCUUUACCCUUUGCCC	CAGACGACUCGCCCGA	NONE
8.40(1) 49	GGGAGGACGAUGCGG	----CCCAUUAGUCCUCAUUAGU-----CCCCUGUGCCC	CAGACGACUCGCCCGA	
8.41(1) 50	GGGAGGACGAUGCGG	CAUCUUAUCCUCCAUCAGUUACUCUCGUUAUUCCCGCCC	CAGACGACUCGCCCGA	
8.45(1) 51	GGGAGGACGAUGCGG	UCC-AAAUCCUCUCCAUGUUAGCAUUCAGCCUUGUCCC	CAGACGACUCGCCCGA	
8.46(1) 52	GGGAGGACGAUGCGG	-UUCCGACAAUUCCUCCACCAUUAGAAUUCUUGCUGCCC	CAGACGACUCGCCCGA	
8.47(1) 53	GGGAGGACGAUGCGG	UCUUGAUCCUCCUUUGUGUCUUUUGUCUUCCCUGCCC	CAGACGACUCGCCCGA	
8.48(2) 54	GGGAGGACGAUGCGG	AAGUAAACGUUGAAGUAAAAAUUCGUUCUCUCGGUAUU-GGC	CAGACGACUCGCCCGA	TGFβ2
8.49(1) 55	GGGAGGACGAUGCGG	-UCCGAUCAGUUCCUUCGAUUAAUCUUCUUUCCUGCCCCC	CAGACGACUCGCCCGA	
8.51(1) 56	GGGAGGACGAUGCGG	AAUCCUUCUCCUGAUGAAUAUGACCUUUUUCUUGCUCCC	CAGACGACUCGCCCGA	
8.52(1) 57	GGGAGGACGAUGCGG	AUGAUCUUUAAUGUCUGGUUUGGAGGUCAAUGCGGGUGCCC	CAGACGACUCGCCCGA	
8.56(1) 58	GGGAGGACGAUGCGG	AGAUGGUACUCCAUCUCCUUUAUGUGCCCAUCGCUGUCCC	CAGACGACUCGCCCGA	
8.57(1) 59	GGGAGGACGAUGCGG	UCCUC-GAUUCU------AAUUUACUCCUUUUUCCCC	CAGACGACUCGCCCGA	
8.61(1) 60	GGGAGGACGAUGCGG	UCUACCCUUUAGCAGUAUUUGUUUCCAUCGUUGUUGCCC	CAGACGACUCGCCCGA	
8.62(1) 61	GGGAGGACGAUGCGG	-CACAAUAUUCUCCUCACUUCCAGUAUUUUCCUGUCCC	CAGACGACUCGCCCGA	
8.64(1) 62	GGGAGGACGAUGCGG	UCCUCAACCUUAGACUUUCAUUUCCUUCAGUUCCUCUGCCC	CAGACGACUCGCCCGA	
8.65(1) 63	GGGAGGACGAUGCGG	UAGUGGUCUGUCAAAGGAAUAGCUAGUAGUGUUUGGUCCC	CAGACGACUCGCCCGA	
8.69(1) 64	GGGAGGACGAUGCGG	CAUCUCCUUAGCAUACCAGUAGUUUAUCCCUUGUCCC	CAGACGACUCGCCCGA	
8.71(1) 65	GGGAGGACGAUGCGG	AGCGACAGUAUAGUAGUACUCUAGUGCUGUCUGUCCC	CAGACGACUCGCCCGA	
8.72(1) 66	GGGAGGACGAUGCGG	ACCUCUCAUGAUCAGCAUCUCGCGUAAUCACGGUUCACCC	CAGACGACUCGCCCGA	
8.74(1) 67	GGGAGGACGAUGCGG	UCCGUACUCCAUUUCUAUUUGAUCCUUUUCUUCUCUGCCC	CAGACGACUCGCCCGA	
8.75(1) 68	GGGAGGACGAUGCGG	AACCCACGACCUUACCUUAAAUCAUGUAAUUCUUCUCUGCCC	CAGACGACUCGCCCGA	
8.76(1) 69	GGGAGGACGAUGCGG	------AGAUAAUGAGUGACGGUGAUUAUAGAGUCUGCCC	CAGACGACUCGCCCGA	
8.79(1) 70	GGGAGGACGAUGCGG	UUCCUCAAUUCUUCCAUCUUCAUAAAUGUUUCCCUUUGCCC	CAGACGACUCGCCCGA	
8.80(1) 71	GGGAGGACGAUGCGG	UUCCU------UCCAACGUUAUCUACUUUCU----GCCC	CAGACGACUCGCCCGA	

a Names are given in the form Round 8.clone number followed by the number of clones of that sequence that were isolated in parentheses.

b -, gaps introduced to designate sequences with selected regions that are shorter than 40 bases. An attempt was made to align such sequences with other sequences but the alignment is not necessarily optimal.

Underlined bases are those that differ from the ligand 14i-1 (**Table 7**). A=2'-OH A; C=2'-F C; G=2'-OH G; U=2'-F U.

c FILTER, filter-binding sequence; NONE, no binding to TGFβ2 or filters, TGFβ2, binds to TGFβ2 as well as ligand 14i-1

62

Table 6. Conditions and results of resonant mirror (rm) optical biosensor SELEX.

Progress of IASYS SELEX with TGFβ2

Rd	TGFβ2, Arcsec[1]		[RNA], µM[2]	Vol, µL[3]	Binding (min)[4]	Dissociation (min)[5]	Elution[6]
	C1	C2					
10	1777	0	1	50	27	29	water
11	1777	0	10	50	30	60	water
12	1777	0	10	50	60	150	water
13	1893	0	0.05	50	37	73	water&SDS
14	1721	0	3.5	50	30	35	water&SDS

[1]Amount of TGFβ2 immobilized expressed in Arcsec where 1 Arcsec is 5 pg/mm^{2} protein. The protein is immobilized in an area of 4 mm[2] in cell 1 (C1).
[2]Concentration of RNA pools
[3]Volume of RNA solution used
[4]Length of binding phase in min
[5]Length of dissociation phase in min
[6]Elution used

Table 7. Sequences isolated from round 13 of resonant mirror SELEX

NAME[a]	SEQ ID NO.	SEQUENCE[b]		
14i-1	72	GGGAGGACGAUGCGG	AAGUAACGUUGUAGUAAAAUUCGUUCUCUCGG-CAUUUGGC	CAGACGACU-CGCCCGA
13.20(1)	73	GGGAGGACGAUGCGG	AAGUAACGUUAUAGUAAAAUUCGUUCUCUCGG-UAUU_GGC	CAGACGACU-CGCCCGA
13.22(2)	74	GGGAGGACGGUGCGG	AAGUAACGUUGUAGUAAAAUUCGUUCUCUCGG-CGUUUGGC	CAGACGACU-CGCCCGA
13.24(2)	75	GGGAGGACGAUGCGG	AAGUAACGUUGUAGUAAAAUUCGUUCUCUCGG-CGUUUGGU	CAGACGACU-CGCCCGA
13.30(1)	76	GGGAG_ACGAUGCGG	AAGUAACGUUGUAGUAAAAUUCGUUCUCUCGG-CAUUUGGC	CAGACGACU-CGCCCGA
13.32(1)	77	GGGAGGACGAUGCGG	AAGUAACGUUGUAGAAGUAAAAUUCGUUCUCUGG-CGUUUGGU	CAGACGACU-CGCCCGA
13.34(1)	78	GGGAGGACGAUGCGG	AAGUAACGUUGAAGUAAAAUUCGUUCUCCUGG-UA_UUGGC	CAGACGACU-CGCCCGA
13.36(2)	79	GGGAGGACGAUGCGG	AAGUAACGUUGAAGUAAAAUUCGUUCUCUCGG-CAUUUGGC	CAGACGACU-CGCCCGA
13.40(1)	80	GGGAGGACGAUGCGG	AAGUAACGUUGUAGUAAAAUUCGUUCUCUGG-CAUUU_GC	CAGACGACU-CGCCCGA
13.42(1)	81	GGGAGGACGAUGCGG	AAGUAACGUUAAAGUAAAAUUCGUUCUCUCGG-CGUUUGGC	CAGACGACU-CGCCCGA
13.44(1)	82	GGGAGGACGAUGCGG	AAGUAACGUUGAAGUAAAAUUCGUUCUCUCGG-CGUUUGGC	CAGACGACU-CGCCCGA
13.48(1)	83	GGGAGGACGAUGCGG	AAGUAACGUUGUAGUAAAAUUCGUUCUCUCGG-UAUUUGGC	CAGACGACU-CGCCCGA
13.50(1)	84	GGGAGGACGAUGCGG	AAGUAACGUUGUAGUAAAAUUCGUUCUCUUGG-UCUU_GGC	CAGACGACU-CGCCCGA
13.54(1)	85	_GGAGGACGAUGCG_	AAGUAACGUUGUAGUAAAAUUCGUUCUCUCGGGCAUUUGG_	CAGACGACUCGCCCGA

a Names are given in the form Round 13.clone number followed by the number of clones of that sequence that were isolated. The sequence of 14i-1 is shown at the top for comparison.

b Underlined bases are those that differ from ligand 14i-1 from the filter SELEX. A=2'-OH A; C=2'-F C; G=2'-OH G; U=2'-F U.

64

Table 8. Sequences and boundaries of TGFβ2 ligands isolated from rounds 14 and 21 of filter SELEX.

Name[a]	SEQ ID NO:	SEQUENCE[b]	Kd (nM)	Ki (nM)
14i-1	72	GGGAGGACGAUGCGGAAGAGUAAACGUUGUAGUAAAAUUCGUUCUCUCGGCAUUUGGCCAGACGACUCGCCCGA	10	230
21a-4	86	GGGAGGACGAGUCGGGCGUUGUUUAGUCGUAUAUAAGUCCGCUUGUCCCCAGACGACUCGCCCGA	3	30
21a-21	87	GGGAGGACGAUGCGG-UUCAGGAGGUUAUUACAGAGUCUGUAUAGCUGUACUCCCCAGAGACGACUCGCCCGA	1	10
region:		5' fixed selected 3' fixed		

[a] Names are in the form: round sequence was isolated-clone number.

[b] Boundaries are underlined. Fixed regions are in bold-faced type. Selected sequences are in plain type.

A=2'-OH A; C=2'-F C; G=2'-OH G; U=2'-F U

Table 9.   Number of sequences isolated using the SELEX process.

Sequence	SELEX round							TOTAL
	8-spr	13-rm	14i	16a	18b	21a		
14i-1	0	0	75	2	0	0		77
14i-1 variants	21	15	22	2	0	0		60
21a-4	0	0	0	0	0	3		3
21a-4 variants	0	0	4	7	0	2		13
21a-21	0	0	0	1	11	38		50
21a-21 variants	0	0	0	2	4	4		10
unidentified	36	0	0	0	0	0		36
filter-binding	12	0	1	1	0	1		15
TOTAL	69	15	102	15	15	48		264

Table 10.   Characteristics of nucleic acid pools isolated using the SELEX method.

Round[a]	Sequence of pool[b]	% of pool[c]	% of transformants[d]	% of clones[e]
0	random	14i-1: <0.03		
6-spr	random	14i-1: ~1		
8-spr	slightly nonrandom	14i-1: ~5		14i-1: 30 other: 70
9-spr	nonrandom			
9-rm	can read sequence of ligand 14i-1			
10-rm	can read sequence of ligand 14i-1			
11-rm	can read sequence of ligand 14i-1			
12-rm	can read variants of ligand 14i-1 sequence			
13-rm	can read variants of ligand 14i-1 sequence	14i-1: 10-100 21a-21: <0.1		14i-1: 100
14i		21a-21: 0.2-0.5		14i-1: 93 21a-4: 4 21a-21: 0 other: 3
16a		21a-21: 3-100		14i-1: 27 21a-4: 47 21a-21: 20 other: 6
18b		21a-21: 3-100		21a-21: 100
21a		21a-21: 3-100	21a-4: 9 21a-21: 90 other: 1	21a-4: 10 21a-21: 84 other: 6

Table 10. (Continued)

[a] spr, from surface plasmon resonance biosensor SELEX; rm, from resonant mirror optical biosensor SELEX.
[b] Determined by primer extension of bulk nucleic acid pools with 3'N7 primer.
[c] Determined by RT-PCR of bulk nucleic acid pools with a ligand-specific primer.
[d] Determined by PCR of individual transformants with a ligand-specific primer.
[e] Determined by sequencing of clones. Includes sequence variants of ligands.

Table 11. Truncates of human TGFβ2 nucleic acid ligand 21a-21.

NAME	SEQUENCE[a]	SEQ ID NO:	BINDING[b]	LENGTH[c]	BIO-ACTIVITY[d]
21a-21	GGGAGGACGAUGCGGUUCAGGAGGAGGGUUAUUACAGAGAGUCUGUAUAGCUGUAACUCCCCAGAGACGACUCGCCCGA	87	0.5	70	1
21a-21 (U6G)	GGGAGGACGAUGCGGUUCAGGAGGAGGGUUAUUACAGAGAGUCUGUAUAGCUGUAACUCCCCAGAGACGACUCGCCCGA	88	250	34	
21a-21Δ5'	GGUUCAGGAGGAGGGUUAUUACAGAGAGUCUGUAUAGCUGUAACUCCCCAGAGACGACUCGCCCGA	89	0.5	56	
21a-21Δ3'	GGGAGGACGAUGCGGUUCAGGAGGAGGGUUAUUACAGAGAGUCUGUAUAGCUGUAACUCCCCA	90	100	56	
21a-21Δ5',3'	GGUUCAGGAGGAGGGUUAUUACAGAGAGUCUGUAUAGCUGUAACUCCCCA	91	0.5	42	1
21a-21 (ML-94)	GGAGGUUAUUACAGAGAGUCUGUAUAGCUGUACUCCCC	92	0.5	36	
21a-21 (ML-95)	GGAGGUUAUUACAGAGAGUCUGUAUAGCUGUACUCC	93	1	34	
21a-21 (ML-96)	GGAGGUUAUUACAGAGAGUCUGUAUAGCUGUA	94	1000	30	
21a-21 (ML-97)	GGAGGUUAUUACAGAGAGUCUGUAUAGC	95	1000	26	
21a-21 (ML-99)	GGAGGUUAUUACAGAGAGUCUGUAUAGC CUCC	96	1000	30	
21a-21 (ML-101)	GGAGGUUAUU AGAGUCU AUAGCUGUACUCC	97	1000	30	
21a-21 (ML-102)	GGAGGUUAUU AGAGUCU AUAGC CUCC	98	1000	26	
21a-21 (ML-103)	GGAGGUUAUUACAGAGUCUGUAUAGCUGUACUC	99	50	33	

Table 11. (Continued)

NAME	SEQUENCE[a]	SEQ ID NO:	BINDING[b]	LENGTH[c]	BIO-ACTIVITY[d]
21a-21(ML-104)	GGAGGUAUUACAGAGAGUCUGUAAGCUGUACU	100	70	32	
21a-21(ML-105)	GGAGGUAUUACAGAGAGUCUGUAAGCUGUAC	101	1000	31	
21a-21(ML-114)	GGAGGUAUUACAGAGAGUCUGUAUAGC GUACUCC	102	1000	33	
21a-21(ML-115)	GGAGGUAUUACAGAGAGUCUGUAUAGCUGU CUCC	103	1000	33	
21a-21(ML-116)	GGAGGUAUUACAGAGAGUCUGUAUAGCU ACUCC	104	1000	32	
21a-21(ML-118)	GGAGGUAU ACAGAGUCUGUAUAGCUGUACUCC	105	1000	33	
21a-21(ML-120)	GGAGGUAUUACAGA UCUGUAUAGCUGUACUCC	106	1000	33	
21a-21(ML-122)	GGAGGUAUUACA AGU UGUAUAGCUGUACUCC	107	1000	32	
21a-21(ML-128)	GGAGGUAUUACAGAGU UGUAUAGCUGUACUCC	108	1000	33	
21a-21(ML-130)	GG GGUUAUUACAGAGUCUGUAUAGCUGUAC CC	109	2	32	
21a-21(ML-132)	GGAGGUAUUAC GAGUCUGUAUAGC GUACUCC	110	1000	32	
21a-21(ML-134)	GGAGA UAUUACAGAGUCUGUAUAGCUGUACUCC	111	10	33	
21a-21(ML-136)	GG GGUUAUU CAGAGUCUGUAUAGCUG AC CC	112	10000	30	
21a-21(ML-138)	GG GGUUAUUA AGAGUCUGUAUAGCU UAC CC	113	10000	30	

Table 11. (Continued)

NAME	SEQUENCE[a]	SEQ ID NO:	BINDING[b]	LENGTH[c]	BIO-ACTIVITY[d]
NX22283	GGAGGUUAUUACAGAGAGUCUGUAUAGCUGUACUCCCC[3'T]	114	0.6	36	0.5
NX22284	GGAGGUUAUUACAGAGUCUGUAUAGCUGUACUCC [3'T]	115	1	34	1
NX22285	GGAGGUUAUUACAGAGUCUGUAUAGCUGUACUCCCCA	116	2	37	
NX22286	GGAGGUUAUUACAGAGUCUGUAUAGCUGUA	117	130	30	>20
NX22301	GAGGUUAUUACAGAGUCUGUAUAGCUGUACUCC [3'T]	118	1	33	2
NX22302	AGGUUAUUACAGAGUCUGUAUAGCUGUACUCC [3'T]	119	100	32	
NX22303	GGUUAUUACAGAGUCUGUAUAGCUGUACUCC [3'T]	120	>100	31	>100
NX22323	PEG-GGAGGUUAUUACAGAGUCUGUAUAGCUGUACUCC [3'T]	121	nt	34	3

[a] The fixed regions are indicated by bold-faced letters. The point mutant in 21a-21(U6G) is underlined and in bold type. A=2'-OH A; C=2'-F C; G=2'-OH G; U=2'-F U
The italicized G at the 5' end of the 5' RNase H cleavage products indicates that ~50% of the time cleavage leaves 2 G's and 50% of the time one G is left. The boundaries in 21a-21 are underlined
[b] Binding is expressed as the ratio of the K_d of ligand /K_d of NX22284. The K_d of NX22284 is ~2 nM.
[c] Length is given in bases.
[d] Bioactivity is expressed as the ratio of the K_i of ligand /K_i of NX22284. The K_i of NX22284 is ~10 nM.

Table 12.   Alignment of human transforming growth factor β amino acid sequences.

							SEQ ID NO.
TGFß1:	ALDTNYCFSS	TEKNCCVRQL	YIDFRKDLGW	KWIHEPKGYH	ANFCLGPCPY	IWSLDTQYSK 60	122
TGFß2:	ALDAAYCFRN	VQDNCCLRPL	YIDFKRDLGW	KWIHEPKGYN	ANFCAGACPY	LWSSDTQHSR 60	123
TGFß3:	ALDTNYCFRN	LEENCCVRPL	YIDFRQDLGW	KWVHEPKGYY	ANFCSGPCPY	LRSADTTHST 60	124
TGFß2 specific:	AA	VQD L	KR	N	A A	S R	

TGFß1:	VLALYNQHNP	GASAAPCCVP	QALEPLPIVY	YVGRKPKVEQ	LSNMIVRSCK	CS 112	125
TGFß2:	VLSLYNTINP	EASASPCCVS	QDLEPLTILY	YIGKTPKIEQ	LSNMIVKSCK	CS 112	126
TGFß3:	VLGLYNTLNP	EASASPCCVP	QDLEPLTILY	YVGRTPKVEQ	LSNMVVKSCK	CS 112	127
TGFß2 specific:	S I	S		I K I			

Table 13. Truncates of human TGFβ2 nucleic acid ligand 14i-1.

NAME	SEQUENCE[a]	SEQ ID NO.	BINDING[b]	LENGTH[c]
14i-1	GGGAGGACGAUGCGGAAGUAACGUUGUAGUAAAAUUCGUUCUCUCGGCCAUUUGGCCAGACGACUCGCCCGA	72	1	71
14i-1Δ5'd	GGAAGUAACGUUGUAGUAAAAUUCGUUCUCUCGGCCAUUUGGCCAGACGACUCGCCCGA	128	>100	56
14i-1Δ3'd	GGGAGGACGAUGCGGAAGUAACGUUGUAGUAAAAUUCGUUCUCUCGGCAUUUGGCCA	129	3	57
14i-1Δ5,3'd	GGAAGUAACGUUGUAGUAAAAUUCGUUCUCUCGGCAUUUGGCCA	130	>100	42
14i-1t5-41	gGGAgGAUGCGGAAGUAACGUUGUAGUAAAAUUCcUUC	131	1	38
14i-1t5-38	gGGAgGAUGCGGAAGUAACGUUGUAGUAAAAUUCc	132	>100	35
14i-1t5-35	gGGAgGAUGCGGAAGUAACGUUGUAGUAAAAU	133	>100	32
14i-1 (ML-86)	gGGAgGAUGCGGAAGUAACGUAACGUUGUAGU UCcUUC	134	>100	33
14i-1 (ML-87)	gGGAgGAUGCGGAAGUAACGUAACGUUGUAGU	135	>100	27
14i-1 (ML-89)	gGgaGgAGUAACGUUGUAGU	136	>100	20

a   Lowercase letters indicate bases not found at that position in the full length ligand that were added or changed to maintain transcriptional efficiency. Boundaries are underlined. The fixed regions are in bold-faced type. The italicized G at the 5' end of the 5' RNase H cleavage products indicates that ~50% of the time cleavage leaves 2 G's and 50% of the time one G is left.
A=2'-OH A; C=2'-F C; G=2'-OH G; U=2'-F.

b   Binding is expressed as the ratio of K_d (ligand)/Kd (14i-1). The K_d of 14i-1 is ~10 nM.

c   Length is in bases.

d   Produced by RNase H digestion.

77

Table 14.   Truncates of human TGFβ2 nucleic acid ligand 21a-4.

Name	Sequence[a]	SEQ ID NO.	Binding[b]	Length[c]
21a-4	**GGGAGGACGAU**GCGGCGUUGUUUAGUCGUAUGUAUAUACUAAGUCCGCUUGU<u>CCCCAGACGACUCGCGCCCGA</u>	86	1	71
21a-4Δ5'[d]	GGCGUUGUUUAGUCGUAUGUAUAUACUAAGUCCGCUUGU<u>CCCCAGACGACUCGCGCCCGA</u>	137	>100	56
21a-4Δ3'[d]	**GGGAGGACGAU**GCGGCGUUGUUUAGUCGUAUGUAUAUACUAAGUCCGCUUGU<u>CCCCA</u>	138	1	57
21a-4Δ5',3'[d]	GGCGUUGUUUAGUCGUAUGUAUAUACUAAGUCCGCUUGU<u>CCCCA</u>	139	>100	42
21a-4 (ML-91)	ggGgaGCGGCGUUGUUUAGUCGUAUGUAUAUACUAAGUCCGCUU	140	1	44
21a-4 (ML-92)	ggGgaGCGGCGUUGUU gaaa AGUCCGCUU	141	>100	27
21a-4 (ML-108)	ggGgaGCGGCGUUGUUU CGUAUGUAUAU AAGUCCGCUU	142	>100	38
21a-4 (ML-109)	ggGgaGCGGCGUUGUUU AUGUAU AAGUCCGCUU	143	>100	33
21a-4 (ML-110)	ggGgaGCGGCGUUGUUUAGUCGUAUGUAUAUACUAAGUCCGC	144	1	42
21a-4 (ML-111)	ggGgaGCGGCGUUGUUUAGUCGUAUGUAUAUACUAAGU	145	30	38

a ; Lowercase letters indicate bases not found at that position in the full length ligand. Underlining indicates boundary positions. The fixed region sequences are indicated in bold-faced lettering. The italicized G at the 5' end of the 5' RNAse H cleavage products indicates that ~50% of the time cleavage leaves 2 Gs and 50% of the time one G is left. A=2'-OH A; C=2'-F C; G=2'-OH G; U=2-F U

b Binding is expressed as the ratio of K_d (ligand)/K_d (21a-4). The K_d of 21a-4 is ~3 nM.

c Length is expressed in bases.

d These ligands were generated by RNAse H digestion of 21a-4.

Table 15.   Biased SELEX conditions and results.

Round[a]	[RNA][b], Nm	[TGFβ2], nM	RNA[b]/protein	[Competitor]	% Bound	% Background	Bound/background	Kd (nM)[c]
34N7.21a-21 round 0 nucleic acid								
1a	1000	150	7	0	1.4	1.4	1.0	870
2a	450	300	1.5	0	1.7	1.0	1.7	395
3a	10	50	0.2	0	17.5	1.0	17.5	186
4a	50	10	5	0	11.0	0.9	12.3	25
4b	50	10	5	333 nM NX22284	2.2	1.3	1.7	17
5a	8	1	8	0	1.4	0.9	1.5	8
5b	8	1	8	100 nM NX22284	0.8	0.7	1.1	1
6a	4	0.5	8	0	2.9	2.9	1.0	17
6b	6	0.5	12	100 nM NX22284	1.8	1.3	1.4	1
7a	5	0.25	20	0	0.5	0.14	3.4	1
7b	5	0.25	20	200 nM NX22284 5 mM tRNA	0.15	0.1	1.5	0.5
8a	1	0.05	20	0	1.05	1.1	0.9	1
8b	1	0.05	20	100 nM NX22284 5 mM tRNA	0.6	0.5	1.2	3
9a	125	1	125	0	0.6	0.5	1.2	nd
9b	0.9	0.01	90	0	0.15	0.14	1.0	nd

[a] a series, without competitor; b series, with competitors

[b] nucleic acid ligand library

[c] nd, not determined

Table 16. Nucleic acid ligands isolated from round 5a of a human TGFβ2 biased SELEX.

NAME[a]	5' FIXED	SELECTED[b]	3' FIXED	A = SEQ ID NO:	B = CHANGES[c]	C = BINDING[d]
putative structural element: 21a-21:	GGGAGGACGAUGCGG	S1 B S2 L S2 S1 UUCAGGAGGUUAUUACAGAGUCUGUAUUAGCUGUAUAGCUGUACUCCC	CAGACGACUCGCCCGA	72	0	1.0
1: (2)	GGGAGGACGAUGCGG	GGUGAUUAUUACAGAGUAUGUAUAGCUGUACCCC	CAGACGACUCGCCCGA	146	4	0.8
2: (1)	GGGAGGACGAUGCGG	AGGCGUUAUUAGAGAGUCUGUAUAGCUCUAGCCC	CAGACGACUCGCCC-GA	147	7	0.6
4: (1)	GGGAGGACGAUGCGG	GGAGGGUAUUACAGAGUAUGUAUAGCUGUACUCC	CAGACGACUCGCCCGA	148	2	1.4
6: (2)	GGGAGGACGAUGCGG	GGAGGGUAUUAUAGAGUCUGUAUAGCUAUACCCC	CAGACGACUCGCCCGA	149	3	1.6
7: (1)	GGGAGGACGAUGCGG	GAGGGUUAUUAUAGAGUCUGCAUAGCUAUACCCC	CAGACGACUCGCCCGA	150	5	0.3
9: (1)	GGGAGGACGAUGCGG	UGAGAGUAUUACGGAGUAUGUAUAGAGCCGUACCCC	CAGACGACUCGCCCGA	151	7	0.3
10: (1)	GGGAGGACGAUGCGG	GGGCAUUAUUUCAGAGUCUGUAUAGCUGUACCCC	CAGACGACUCGCCCGA	152	6	0.3
11: (2)	GGGAGGACGAUGCGG	GCGGAUUAUCACAGAGUAUGUAUAGCUGUGCCGC	CAGACGACUCGCCCGA	153	8	0.4
13: (1)	GGGAGGACGAUGCGG	UGUGAAUAUUAGAGAGUCUGUAUAGCUCUACCCC	CAGACGACUCGCCCGA	154	7	0.2
14: (1)	GGGAGGACGAUGCGG	CGGGAUUAUUACUGAGUCUGUAUAGCAGUACCCC	CAGACGACUCGCCCGA	155	6	0.4
15: (1)	GGGAGGACGAUGCGG	GUGGAAUAUUACGGAGUCUGUAUAGCCGUACUCC	CAGACGACUCGCCCGA	156	6	0.4
17: (1)	GGGAGGACGAUGCGG	GGGGACUAUUAGUGAGUCUGUAUAGCACUACCCC	CAGACGACUCGCCCGA	157	8	0.8
18: (1)	GGGAGGACGAUGCGG	GUGGAUUAUUACAGGCGUCUGUAUAUCUGUACCCC	CAGACGACUCGCCCGA	158	6	1.0
19: (2)	GGGAGGACGAUGCGG	GCAGGUUAUUACAGAGUCUGUAUAGCUGUACUGC	CAGACGACUCGCCCGA	159	2	1.0
20: (1)	GGGAGGACGAUGCGG	GGUAGAUAUCACUGAGUCUGUAUAGCAGUGCCC	CAGACGACUCGCCCGA	160	9	5.7
21: (2)	GGGAGGACGAUGCGG	AGGGAUUAUUACAGAGUAUGUAUAGCUGUACCCC	CAGACGACUCGCCCGA	161	4	0.7
22: (4)	GGGAGGACGAUGCGG	GUGGAUUAUUACAGAGUCUGUAUAGCUGUACCCC	CAGACGACUCGCCCGA	162	4	1.1
25: (1)	GGGAGGACGAUGCGG	GGGCGUUAUUACAGAGUCUGUAUAGCUGUAGCCC	CAGACGACUCGCCCGA	163	4	1.0
26: (1)	GGGAGGACGAUGCGG	GGUGGUUAUUACACAGAGUAUGUAUAGGUGUACCCC	CAGACGACUCGCCCGA	164	4	3.1
28: (1)	GGGAGGACGAUGCGG	AGGGAAUAUUACAGAGUAUGUAUAGCUGUACCCC	CAGACGACUCGCCCGA	165	6	1.0
29: (1)	GGGAGGACGAUGCGG	GGAGUUUAUUACAGCGGUCUGUAUAUCUGUAGCCC	CAGACGACUCGCCCGA	166	5	1.0

Table 16. (Continued)

NAME[a]	5' FIXED	SELECTED[b]	3' FIXED	A = SEQ ID NO: B = CHANGES[c] C = BINDING[d] A B C
30:(1)	GGGAGGACGAUGCGG	UGAGGUUAUUACAGAGUCUGUAUAGCUGUACUCC	CAGACGACUCGCCCGA	167 1 2.4
34:(1)	GGGAGGACGAUGCGG	GGUGGUUAUUAGAGAGUCUGUAUAGCUCUACGCC	CAGACGACUCGCCCGA	168 4 1.7
35:(1)	GGGAGGACGAUGCGG	GGGGAGUAUUAAAGAGUCUGUAUAGCUUUACCCC	CAGACGACUCGCCCGA	169 6 0.8
36:(1)	GGGAGGACGAUGCGG	GGGAGGAUAUAUAGAGUCUGUAUAGCUAUACCCC	CAGACGACUCGCCCGA	170 4 1.9
invariant:		UAU GU UG AUA C		

[a] Number of clones isolated for each sequence is indicated in parentheses.

[b] Nucleotides that differ from the starting sequence are shown in bold-faced lettering. A=2'-OH A; C=2'-F C; G=2'-OH G; U=2'-F U
Putative structural elements: S1, stem 1; B, bulge; S2, stem 2; L, loop. The sequence of ligand 21a-21 is shown at the top for comparison.

[c] Number of changes from starting sequence.

[d] Binding is expressed as K_d (ligand)/ K_d (21a-21). The K_d of ligand 21a-21 is about 1 nM.

Table 17. Highest and lowest affinity TGFβ2 nucleic acid ligands from biased SELEX.

NAME	5' FIXED	SELECTED[a]	SEQ ID NO.	3' FIXED	BINDING[b]	CHANGES[c]
HIGHEST AFFINITY LIGANDS:						
13:	GGGAGGACGAUGCGG	UGUGAAUAUUAGAGAGUCUGUAUAGCUCUACCCC	154	CAGACGACUCGCCCGA	0.2	7
14:	GGGAGGACGAUGCGG	CGGGAUUAUUACUGAGUCUGUAUAGCAGUACCCC	155	CAGACGACUCGCCCGA	0.4	6
21:	GGGAGGACGAUGCGG	AGGGAUUAUUACAGAGUCUGUAUAGCUGUACCCC	161	CAGACGACUCGCCCGA	0.7	4
35:	GGGAGGACGAUGCGG	GGGGAGUAUUAAAGAGUCUGUAUAGCUUUACCCC	169	CAGACGACUCGCCCGA	0.8	6
putative structural elements:		S1 B S2 L S2 S1				
21a-21:	GGGAGGACGAUGCGGUUCAGGAGGUUAUUACAGAGUCUGUAUAGCUGUACUCCC		72	CAGACGACUCGCCCGA	1.0	0
LOWEST AFFINITY LIGANDS:						
36:	GGGAGGACGAUGCGG	GGAGGAUAUUAUAGAGUCUGUAUAGCUAUACCCC	170	CAGACGACUCGCCCGA	2.0	4
30:	GGGAGGACGAUGCGG	UGAGGUUAUUACAGAGUCUGUAUAGCUGUACUCC	167	CAGACGACUCGCCCGA	2.4	1
26:	GGGAGGACGAUGCGG	GGUGGGUAUUACACAGUAUGUAUAGGUGUACCCC	164	CAGACGACUCGCCCGA	3.1	4
6:	GGGAGGACGAUGCGG	GGAGGUUAUUAUAGAGUCUGUAUAGCUAUACCCC	149	CAGACGACUCGCCCGA	3.3	3
20:	GGGAGGACGAUGCGG	GGUAGAUAUCACUGAGUCUGUAUAUAGCAGUGUCCC	160	CAGACGACUCGCCCGA	5.7	9

invariant: UAU   GU UG AUA   C

[a] Nucleotides that differ from the starting sequence are shown in bold-faced lettering. A=2'-OH A; C=2'-F C; G=2'-OH G; U=2'-F U
Putative structural elements: S1, stem1; B, bulge; S2, stem2; L, loop.
[b] Binding is expressed as K_d (ligand)/K_d (21a-21). The K_d of 21a-21 is 1 nM
[c] Number of changes from starting sequence.

Table 18. Substitution of 2'-OH purines with 2'-OCH₃ purines in NX22284 ligand.

NAME	SEQUENCE[a]	SEQ ID NO:	BINDING[b]	LENGTH[c]	BIOACTIVITY[d]
NX22284	GGAGGUAUUaCAGAGUCUGUAUAGCUGUACUCC [3'T]	115	1	34	1
NX22304	ggaggUUaUUaCagagUCUgUaUagCUgUaCUCC [3'T]	171	>100	34	>100
NX22355	GGAGGUUAUUaCagagUCUgUaUagCUgUaCUCC [3'T]	172	>100	34	>100
NX22356	ggagGUUAUUaCAGAGUCUGUAUAGCUGUACUCC [3'T]	173	1	34	1
NX22357	GGAGgUUaUUaCACAGAGUCUGUAUAGCUGUACUCC [3'T]	174	2	34	10
NX22358	GGAGGUUaUUaCACAGAGUCUGUAUAGCUGUACUCC [3'T]	175	1	34	1
NX22359	GGAGGUAUUaCagagUCUGUaUaGCUGUACUCC [3'T]	176	>100	34	>30
NX22360	GGAGGUAUUaCAGAGUCUGUaUaUAGCUgUaCUCC [3'T]	177	1	34	1
NX22374	GGAGGUAUUaCAGAGUCUGUaUAUAGCUGUACUCC [3'T]	178	25	34	>100
NX22375	GGAGGUAUUaCAGAGUCUGUaUAGCUGUACUCC [3'T]	179	>100	34	>300
NX22376	GGAGGUUAUUaCAGAGUCUGUAUaGCUGUACUCC [3'T]	180	50	34	>100
NX22377	ggaggUUaUUaCAGAGUCUGUaUAGUGUaCUGUaCUCC [3'T]	181	1	34	1
NX22383	ggaggUUaUUaCagagUCUGUaUAUagCUgUaCUCC [3'T]	182	500	34	>100
NX22384	ggaggUUaUUaCagagUCUGUaUaUAUagCUgUaCUCC [3'T]	183	10000	34	>100
NX22417	ggaggUUaUUaCagagUCUGUAUaGUGUaGUagCUgUaCUCC [3'T]	184	1	34	10
NX22420	ggaggUUaUUaCagagUCUGUaUAUagCUgUaCUCC [3'T]	185	1	34	1
NX22421	ggaggUUaUUaCagagUCagagUCUGUaUAUagCUgUaCUCC [3'T]	186	2	34	1
NX22426	ggaga-UAUUaCagagUCUGUAUAUAgCUgUaCUCC [3'T]	187	1	33	25
NX22427	gg-ggUUAUUaCagagUCUGUaUAUAgCUgUaC-CC [3'T]	188	0.3	32	0.7

[a] A, 2'-OH A; C, 2'-F C; G, 2'-OH G; U, 2'-F U; a, 2'-OCH₃ A; g, 2'-OCH₃ G. [3'T] signifies a 3', 3' dT cap.

[b] Binding is expressed as the ratio of the K_d of ligand /K_d of NX22284. The K_d of NX22284 is ~1 nM.

[c] Length is given in bases.

[b] Bioactivity is expressed as the ratio of the K_i of ligand /K_i of NX22284. The K_i of NX22284 is ~10 nM.

Table 19. Truncates and 2'-OCH$_3$ purine modifications of nucleic acid ligand #13 from a biased SELEX.

NAME	SEQUENCE[a]	SEQ ID NO:	BINDING[b]	LENGTH[c]	BIOACTIVITY[d]
NX22385	UGUGAAUAUUAGAGAGUCUGUAUAGCUCUACCCC[3'T]	189	0.4	34	4
NX22386	UgUgaAUaUUaGagagUCUGUAUagCUCUaCCCC[3'T]	190	3000	34	>100
NX22387	UgUgaaUaUUagagagUCUgUAUagCUCUaCCCC[3'T]	191	3000	34	30
NX22424	UgUgAAUAUUaGagagUCUGUAUAgCUCUaCCCC[3'T]	192	0.6	34	>100
NX22425	UgUgaaUAUUagagagUCUGUAUAgCUCUaCCCC[3'T]	193	1.5	34	>100

a A, 2'-OH A; C, 2'-F C; G, 2'-OH G; U, 2'-F U; a, 2'-OCH$_3$ A; g, 2'-OCH$_3$ G. [3'T] signifies a 3', 3' dT cap.
b Binding is expressed as the ratio of the K$_d$ of ligand/K$_d$ of NX22284. The K$_d$ of NX22284 is 2 nM.
c Length is given in bases.
d Bioactivity is expressed as the ratio of the K$_i$ of ligand/K$_i$ of NX22284. The K$_i$ of NX22284 is 10 nM.

80

Table 20.   Pharmacokinetic properties of NX22323 in rats using a noncompartmental
analysis.

Parameter	Units	Estimate
Cmax	(µg/mL)	27.1
AUClast	((µg*min)/mL)	3028.0
AUCINF	((µg*min)/mL)	3058.0
Beta t1/2	(min)	630.9
Cl	(mL/(min*kg))	0.33
MRTINF	(min)	350.4
Vss	(mL/kg)	115.0
Vz	(mL/kg)	298.0

Table 21.   Pharmacokinetic properties of NX22323 in rats using a compartmental
analysis.

Parameter	Units	Estimate	StdError	% Error
Cmax	(µg/mL)	16.3	3.3	20.2
AUCINF	((µg*min)/mL)	2486	274	11.0
Alpha-t1/2	(min)	63.5	19.1	30.2
Beta-t1/2	(min)	467.2	83.2	17.8
A	(µg/mL)	14.63	3.21	21.9
B	(µg/mL)	1.70	0.84	49.1
Cl	(mL/(min*kg)	0.402	0.044	11.0
MRTINF	(min)	360.3	35.6	9.9
Vss	(mL/kg)	144.9	23.1	15.9

TABLE 22. Binding and inhibitory activity of 2'-Omethyl- and Pegyl-modifications of lead TGFβ1 truncate ligand CD70

Name	Sequence	SEQ ID NO:	Binding	Bioactivity
ChD70	GGGUGCCUUUUGCCUAGGUUGUGAUUUGUAACCUUCUGCCCA	216	+++	+++
ChD70-m1	gggUgCCUAGGUUGUGAUUUGUAACCUUCUGCCCA	194	+	
ChD70-m2	GGGUGCCUUUUgCCUaggUUGUGAUUUGUAACCUUCUGCCCA	195	++	
ChD70-m3	GGGUGCCUUUUGCCUAGGUUgaUUUgUAACCUUCUGCCCA	196	+++	
ChD70-m4	GGGUGCCUUUUGCCUAGGUUGUGAUUUGUaaCCUUCUGCCCa	197	++	
ChD70-m5	gGGGUGCCUUUUGCCUAGGUUgaUUUgUAACCUUCUGCCCA	198	+++	
ChD70-m6	GGGUGCCUUUUGCCUAGGUUgaUUUgUAACCUUCUGCCCA	199	+++	
ChD70-m7	GGgUGCCUUUUGCCUAGGUUgUGaUUUgUAACCUUCUGCCCA	200	+++	
ChD70-m8	GGGUgCCUUUUGCCUAGGUUgUGaUUUgUAACCUUCUGCCCA	201	+	
ChD70-m9	GGGUGCCUUUUgCCUAGGUUgUGaUUUgUAACCUUCUGCCCA	202	+	
ChD70-m10	GGGUGCCUUUUGCCUaGGUUgUGaUUUgUAACCUUCUGCCCA	203	+++	
ChD70-m11	GGGUGCCUUUUGCCUAGgUUgUGaUUUgUAACCUUCUGCCCA	204	+++	
ChD70-m12	GGGUGCCUUUUGCCUAGGUUgaUUUgUAACCUUCUGCCCA	205	+++	
ChD70-m13	GGGUGCCUUUUGCCUAGGUUgaUUUgUaACCUUCUGCCCA	206	+++	
ChD70-m14	GGGUGCCUUUUGCCUAGGUUgUGaUUUgUAaCCUUCUGCCCA	207	+++	
ChD70-m15	GGGUGCCUUUUGCCUAGGUUgUGaUUUgUAACCUUCUgCCCA	208	+++	
ChD70-m16	GGGUGCCUUUUGCCUAGGUUgaUUUgUAACCUUCUGCCCa	209	+++	
ChD70-m17	gggUGCCUUUUGCCUaggUUgaUUUgUgaUUUgUaACCUUCUGCCCa3'-3'U	210	+++	+++
ChD70-m18	gggUGCCUUUUGCCUaggUUgUGaUUUgUaACCUUCUGCCCa3'-3'U	211	+++	
ChD70-m19	gggUGCCUUUUGCCUaggUUgaUUUgUaaCCUUCUGCCCC3'-3'U	212	++	–
ChD70-m20	gggUGCCUUUUGCCUaggUUgU-----gUaaCCUUCUGCCCa3'-3'U	213	++	
ChD70-m21	gggUGCCUUUUGCCUaggUUg------UaaCCUUCUGCCCa3'-3'U	214	++	
ChD70-m22	gggUGCCUUUUGCCUaggUU-------aaCCUUCUGCCCa3'-3'U	215	+++	

WE CLAIM:

1.   A purified and isolated non-naturally occurring RNA ligand to TGFβ2 wherein said ligand is selected from the group consisting of the sequences set forth in Tables 5, 7, 8, 11, 13, 14, 16-19 and Figure 9 (SEQ ID NOS:21-108 and 128-193).

2.   A Complex comprised of a TGFβ2 Nucleic Acid Ligand and a Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound.

3.   The Complex of Claim 2 further comprising a Linker between said Ligand and said Non-Immunogenic, High Molecular Weight Compound or Lipophilic Compound.

4.   The Complex of Claim 2 wherein said Non-Immunogenic, High Molecular Weight Compound is a Polyalkylene Glycol.

5.   The Complex of claim 4 wherein said Polyalkylene Glycol is polyethylene glycol (PEG).

6   The Complex of claim 5 wherein said PEG has a molecular weight of about between 10-80 K.

7.   The Complex of claim 6 wherein said PEG has a molecular weight of about 20-45 K.

8.   The Complex of claim 7 wherein said Complex is
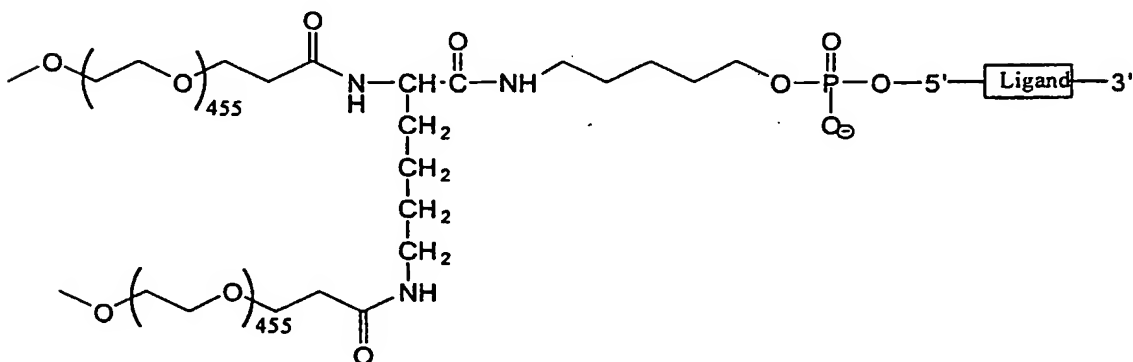
LIGAND=
rGrGrArGrGfUfUrAfUfUrAfCrArGrArGfUfCfUrGfUfUrArGfCfUrGfUrAfCfUfCfC-3'-3'-dT

(SEQ ID NO:115), wherein rG is 2'OH G, rA is 2'OH A, fU is 2'F U and fC is 2'F C.

Fig. 1

Fig. 2A

Fig. 2B

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

14I-1: GGGAGGACGAUGCGGAAGUAACGUUGUAGUAAAAUUCGUUCUCUC | GGCAUUUGGCCAGACGACUCGCCCGA (SEQ ID NO:72)

21a-4: GGGAGGACGA | UGCGGCGUUGUUUAGUCGUAUGUAUAUACUAAGUCCGCUUGUCCCC | AGACGACUCGCCCGA (SEQ ID NO:86)

21a-21: GGGAGGACGAUGCGG-UUCAG | GAGGUUAUUACAGAGUCUGUAUAGCUGUACUCCCC | AGACGACUCGCCCGA (SEQ ID NO:87)

21-4

21-21

14-1
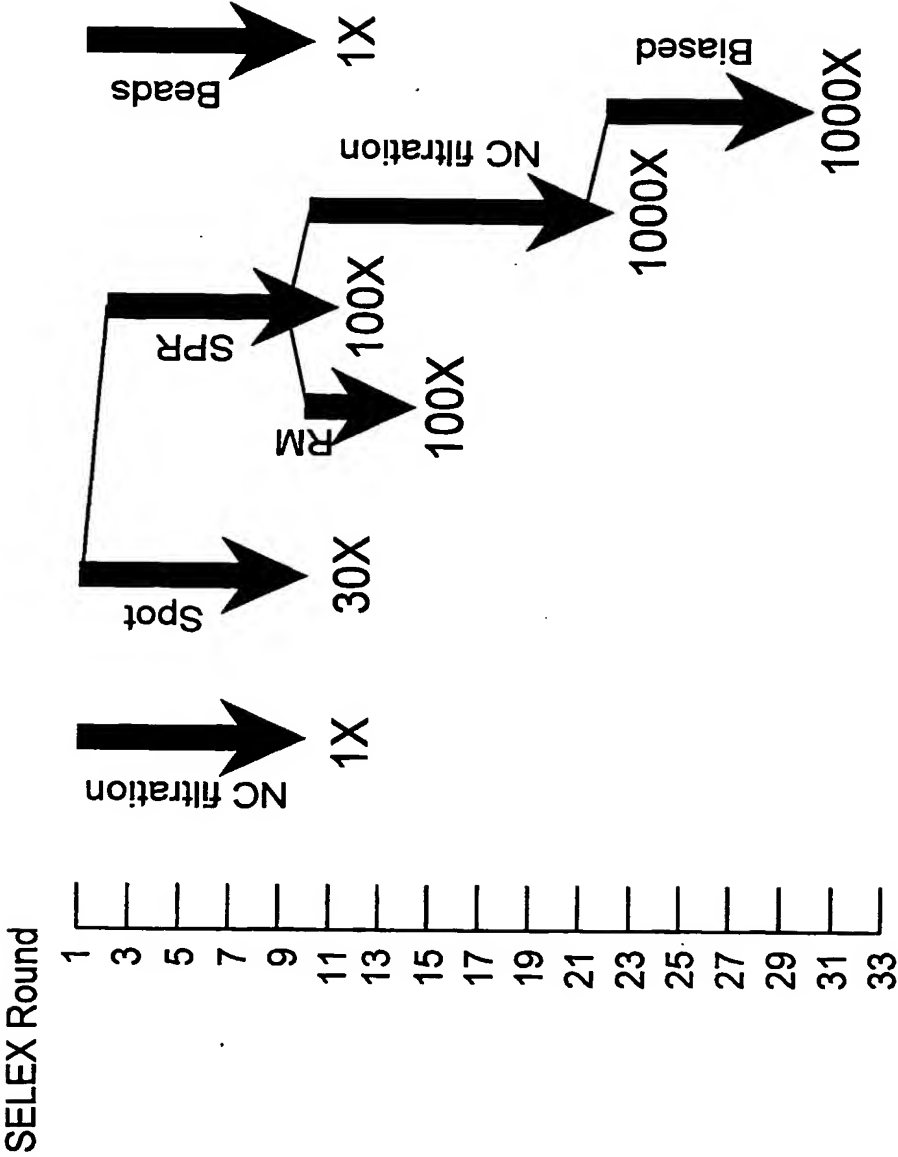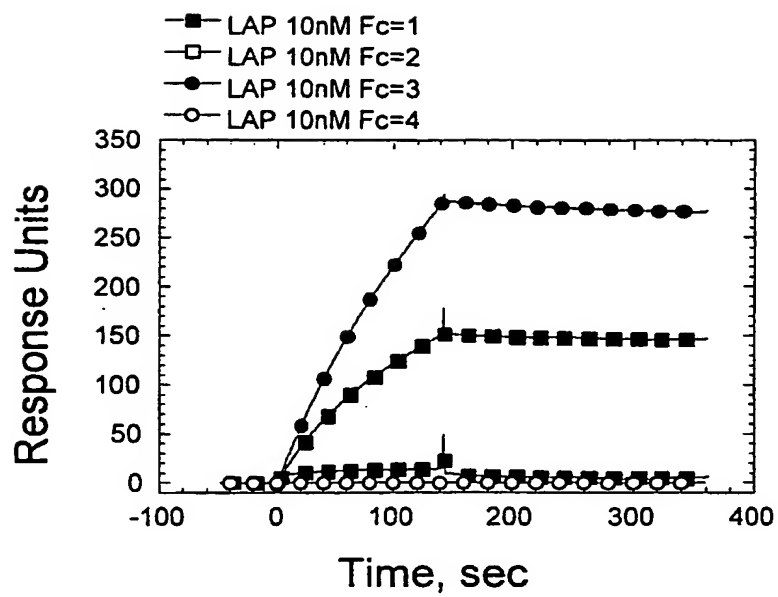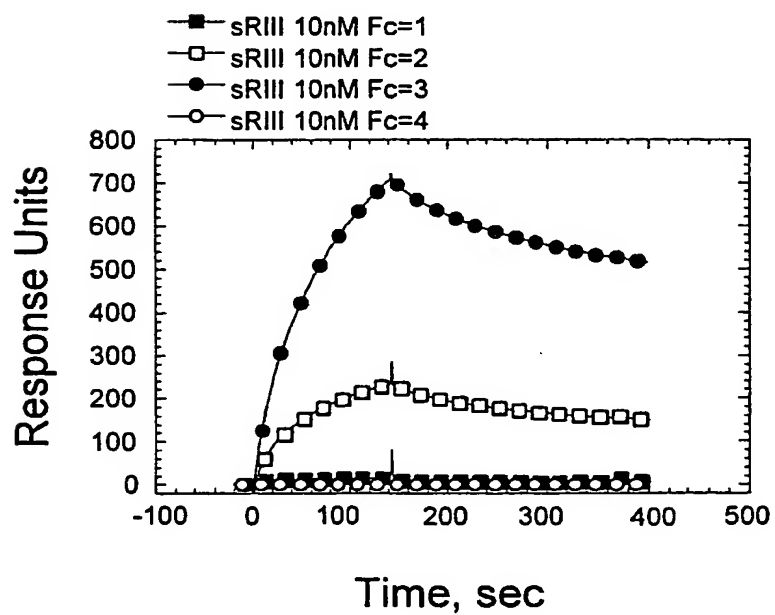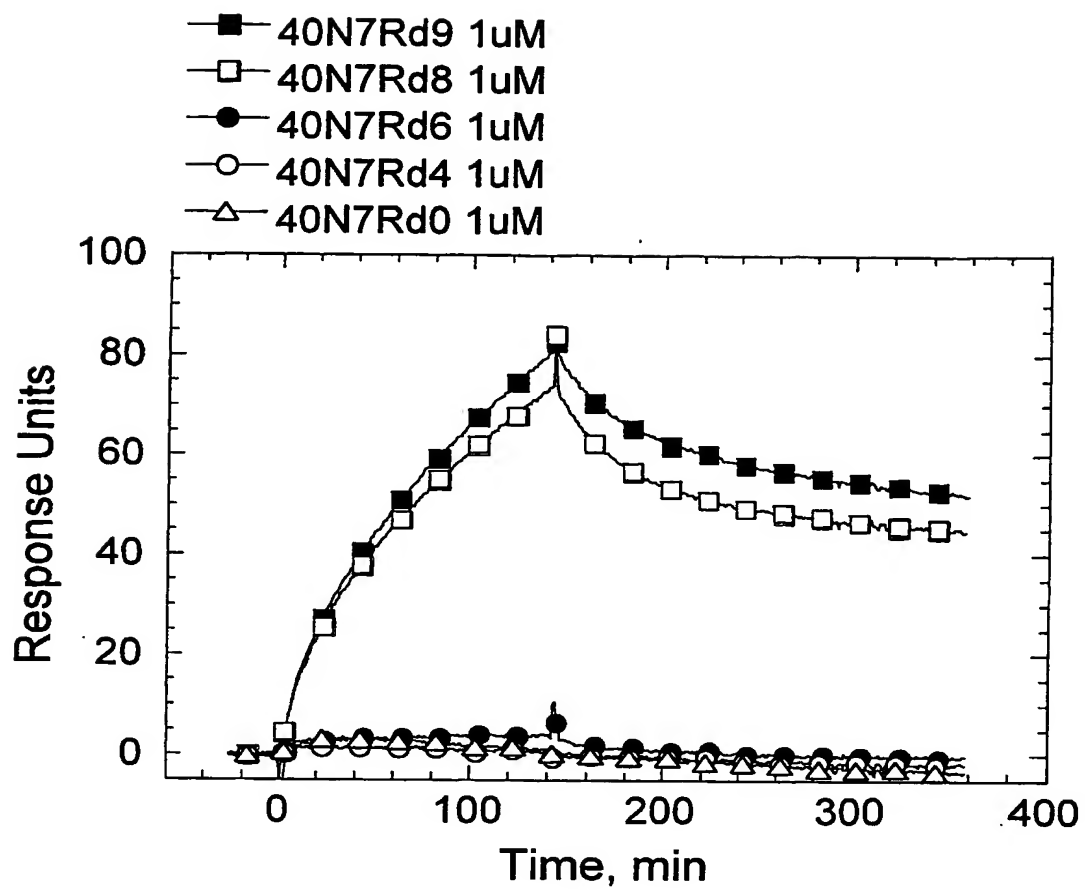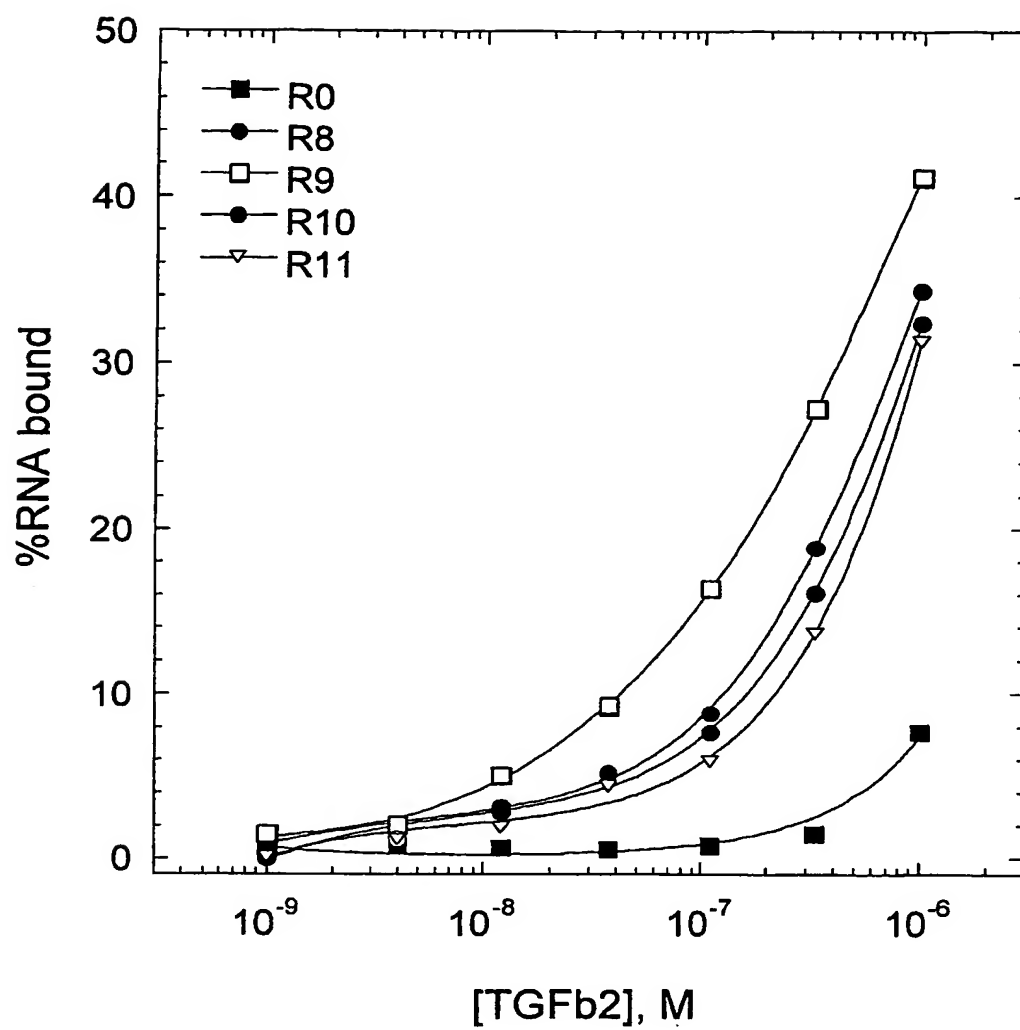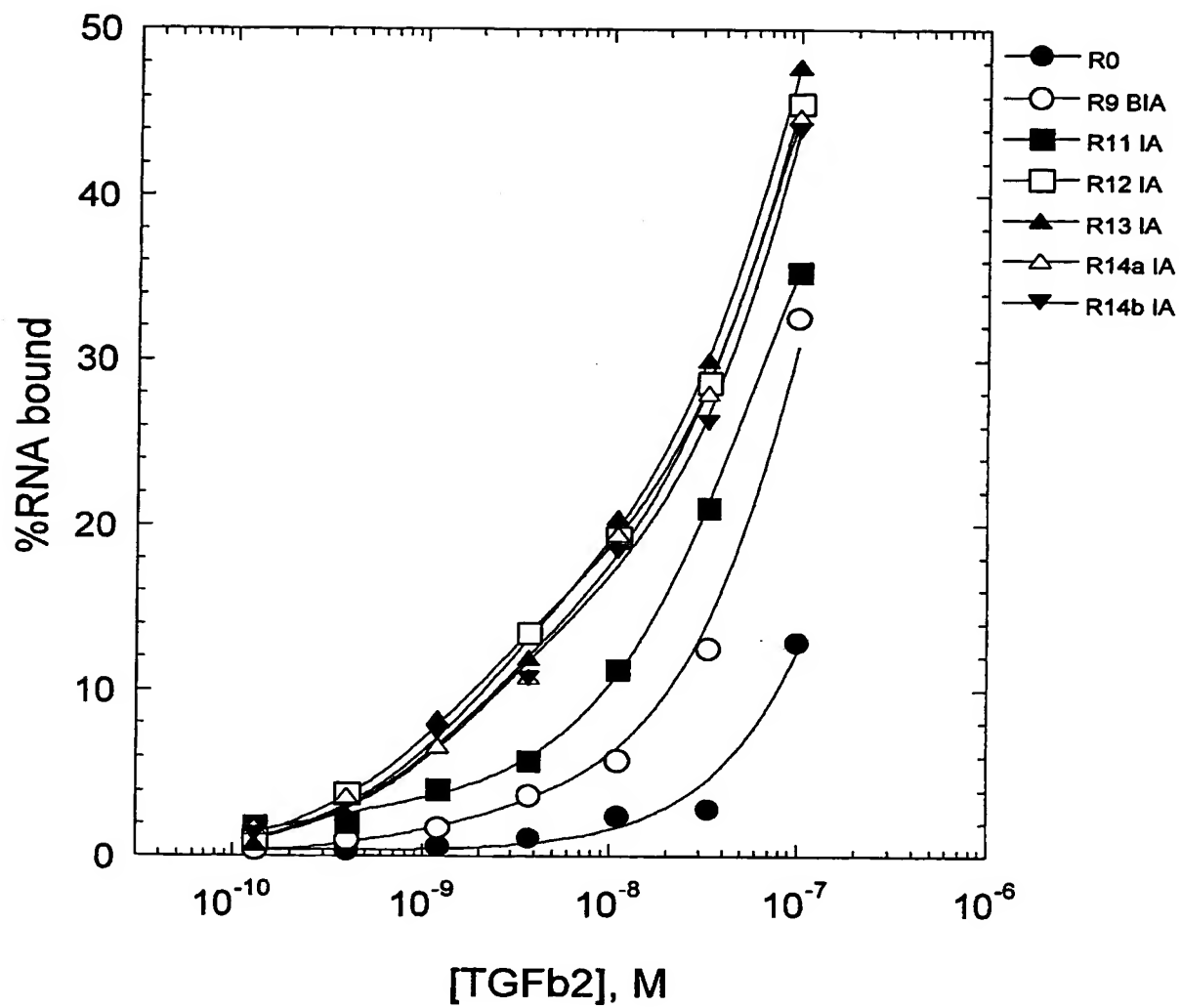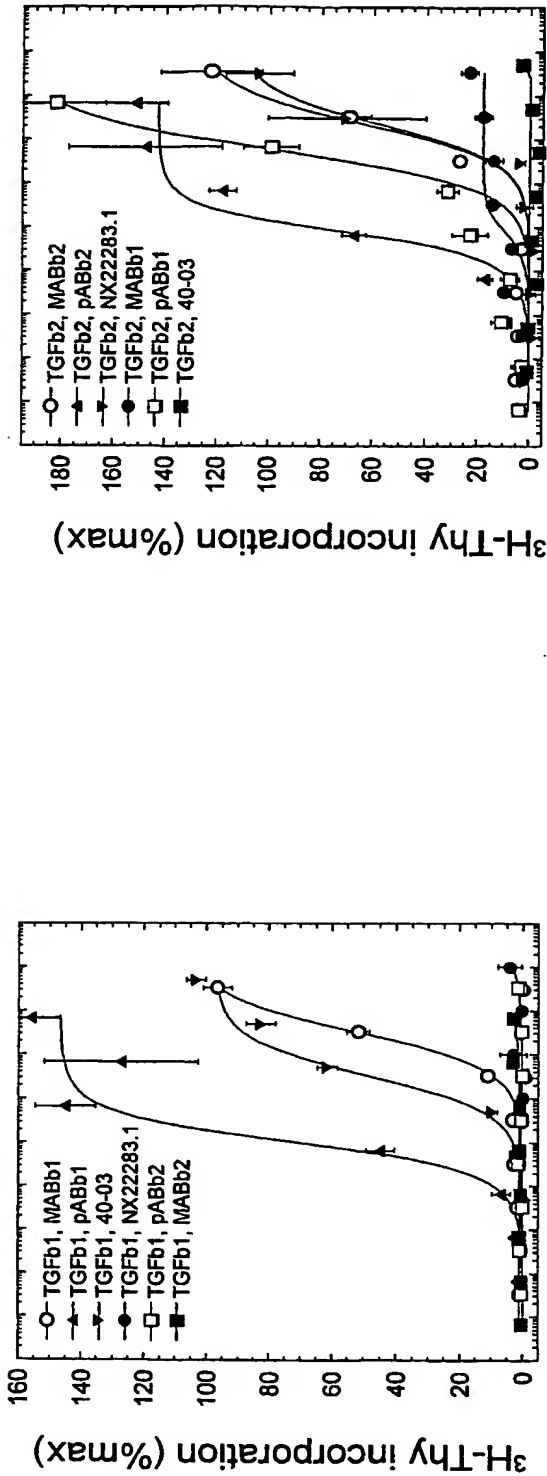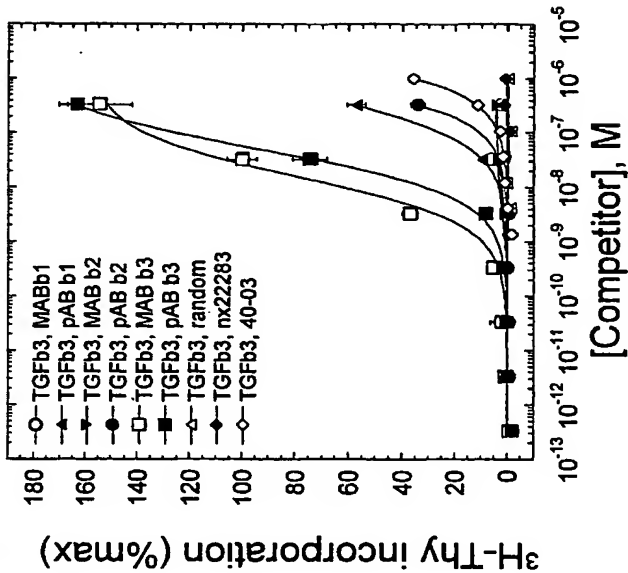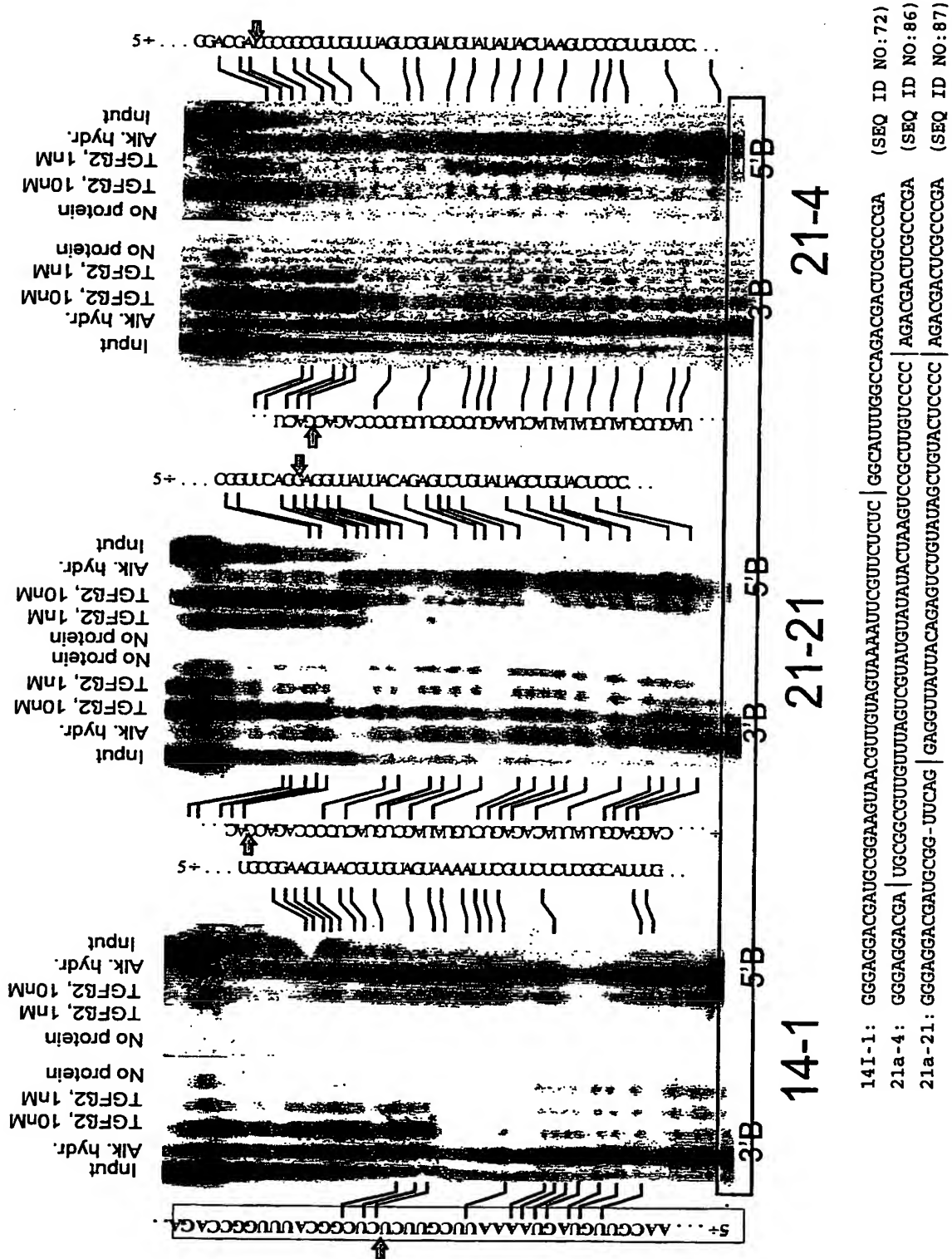
21a-21 (ML-95) or NX22284
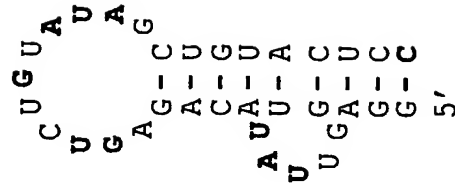
SEQ ID NO:93

21a-4(ML-110)
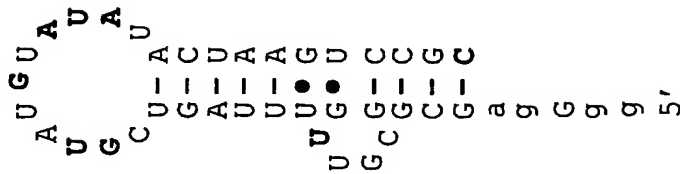
SEQ ID NO:144

14i-1t5-41
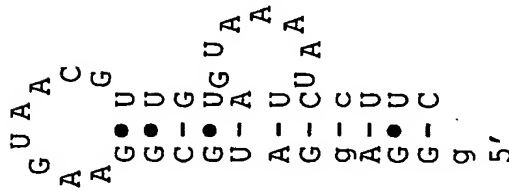
SEQ ID NO:131

Fig. 8
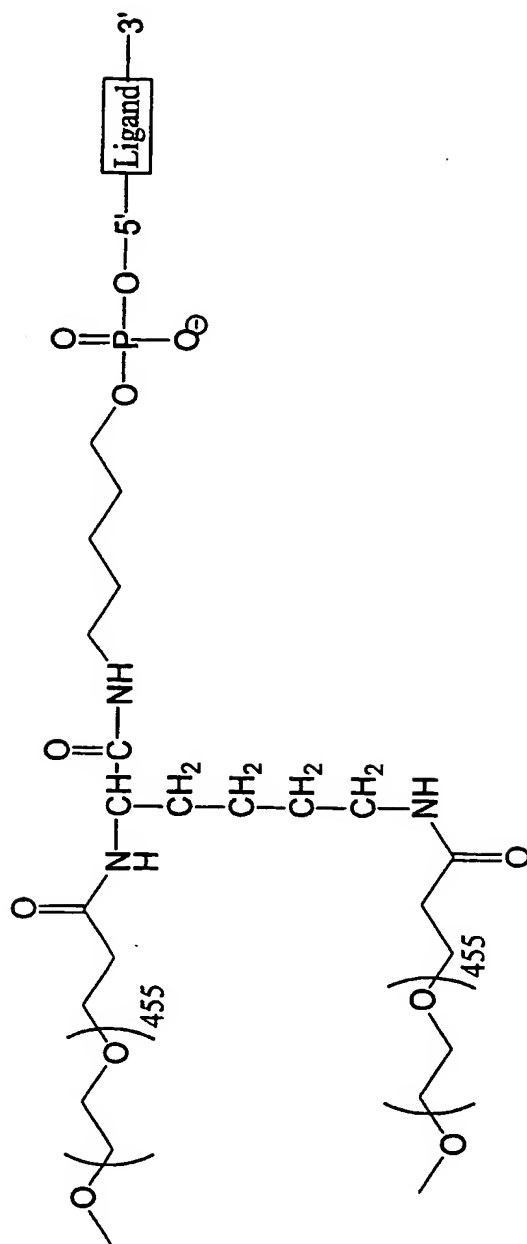
Fig. 9

TGFβ2 LIGAND=
rGrGrArGrGfUrUrAfUfUrAfCrArGrArGfUfCfCrUrGfUfUrArGfCfCrUrGfUrAfCfUfCrC-3'-3'-dT

(SEQ ID NO:115)
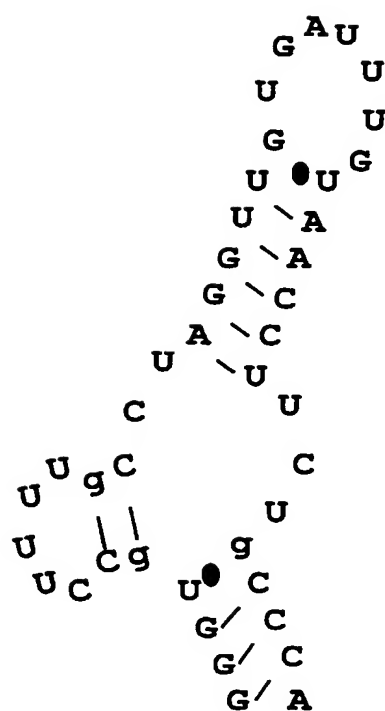
SEQ ID NO:216

Fig. 10
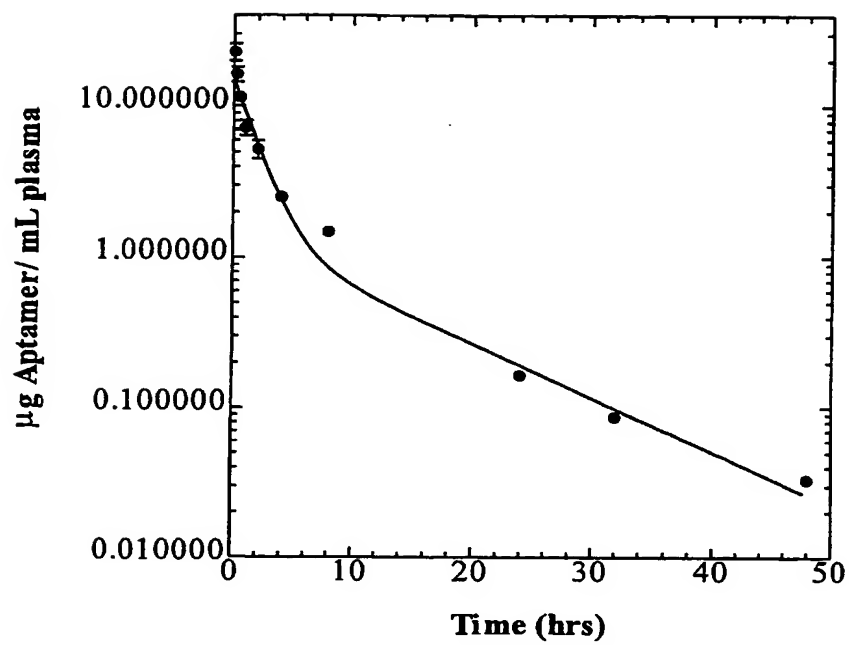
Fig. 11
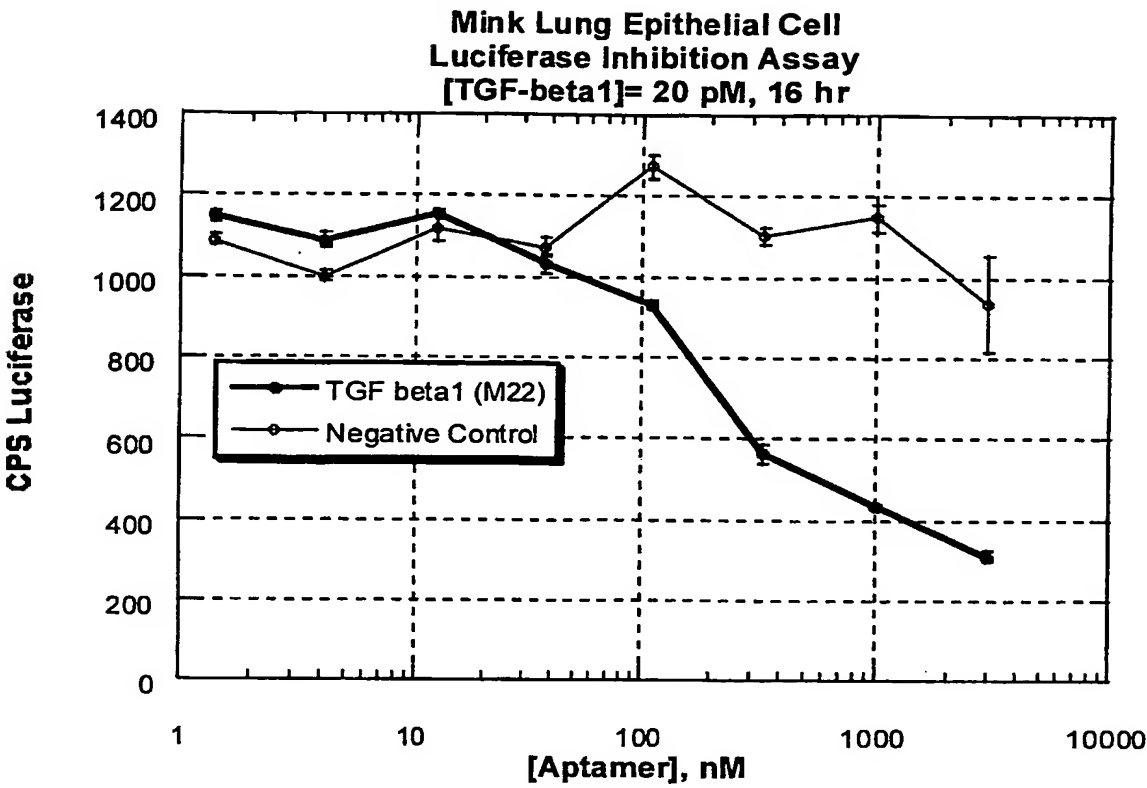
Fig. 12
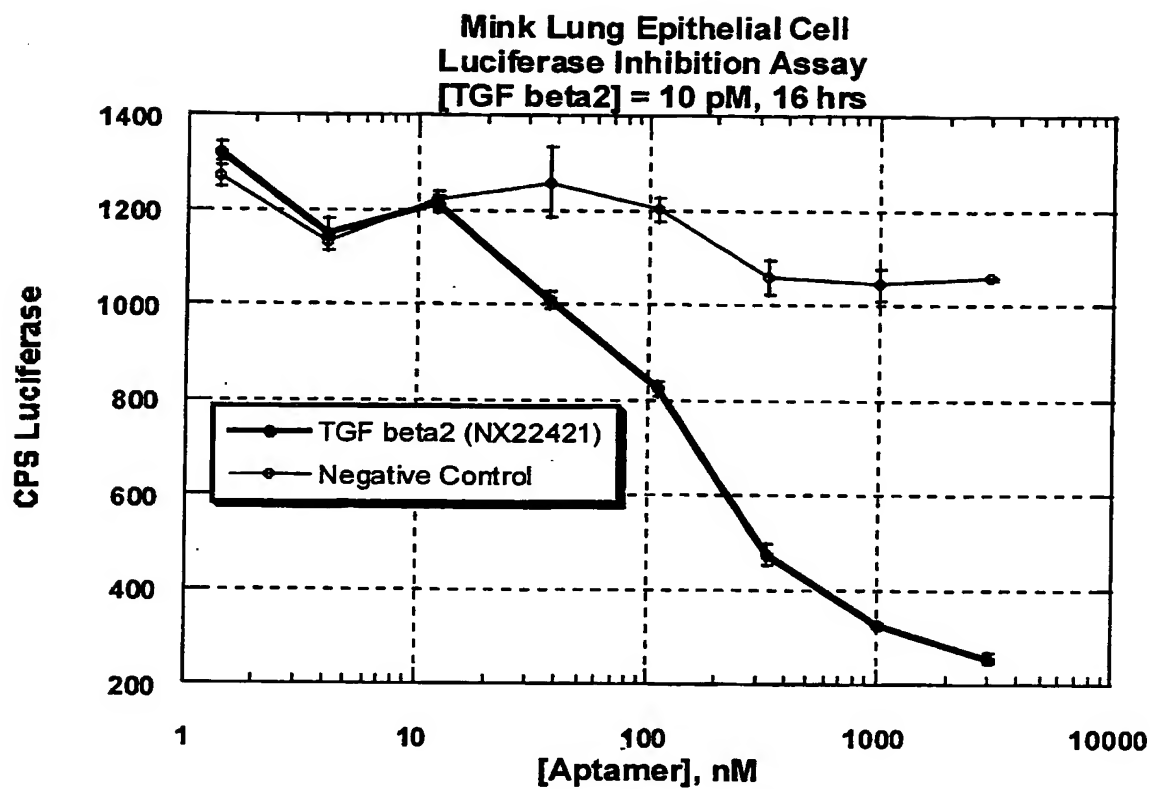
Fig. 13

SEQUENCE LISTING

<110> NeXstar Pharmaceuticals, Inc.

<120> High Affinity TGFBeta Nucleic Acid Ligands and
      Inhibitors

<130> NEX87/PCT

<140>
<141>

<150> 09/046,247
<151> 1998-03-23

<150> 08/458,424
<151> 1995-06-02

<150> 07/714,131
<151> 1991-06-10

<150> 07/931,473
<151> 1992-08-17

<150> 07/964,624
<151> 1992-10-21

<150> 08/117,991
<151> 1993-09-08

<150> 07/536,428
<151> 1990-06-11

<150> 09/363,939
<151> 1999-07-29

<150> 08/434,465
<151> 1995-05-04

<160> 216

<170> PatentIn Ver. 2.0

<210> 1
<211> 71
<212> DNA
<213> Artificial Sequence

```
<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(71)
<223> Feature N at positions 17-56 is A, C, G or T.

<400> 1
tcgggcgagt cgtctgnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnccgc 60
atcgtcctcc c                                                     71

<210> 2
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<400> 2
taatacgact cactataggg aggacgatgc gg                               32

<210> 3
<211> 16
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<400> 3
tcgggcgagt cgtctg                                                 16

<210> 4
<211> 88
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
```

```
<222> (1)..(88)
<223> Feature N at positions 33-72 is A, C, G or T.


<400> 4
taatacgact cactataggg aggacgatgc ggnnnnnnnn nnnnnnnnnn nnnnnnnnnn  60
nnnnnnnnnn nncagacgac tcgcccga                                     88


<210> 5
<211> 88
<212> DNA
<213> Artificial Sequence


<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence


<220>
<221> modified_base
<222> (1)..(88)
<223> Feature N at positions 33-72 is A, C, G or T.


<400> 5
attatgctga gtgatatccc tcctgctacg ccnnnnnnnn nnnnnnnnnn nnnnnnnnnn  60
nnnnnnnnnn nngtctgctg agcgggct                                     88


<210> 6
<211> 71
<212> RNA
<213> Artificial Sequence


<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence


<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F; feature N at positions
      16-55 is A, 2'-FC, G or 2'-FU.


<400> 6
gggaggacga ugcggnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnncagac  60
gacucgcccg a                                                       71


<210> 7
<211> 65
<212> DNA
<213> Artificial Sequence
```

```
<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(65)
<223> Feature N at positions 16-49 is SEQ ID NO: .115
      and A, C, G or T.

<400> 7
gggaggacga tgcggnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnc agacgactcg 60
cccga                                                             65

<210> 8
<211> 82
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(82)
<223> Feature N at positions 33-66 is SEQ ID NO: 115 and
      A, C, G or T.

<400> 8
taatacgact cactataggg aggacgatgc ggnnnnnnnn nnnnnnnnnn nnnnnnnnnn 60
nnnnnncaga cgactcgccc ga                                         82

<210> 9
<211> 82
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(82)
<223> Feature N at positions 33-66 is SEQ ID NO: 115 and
      A, C, G or T.
```

<400> 9
attatgctga gtgatatccc tcctgctacg ccnnnnnnnn nnnnnnnnnn nnnnnnnnnn 60
nnnnnngtct gctgagcggg ct   82

<210> 10
<211> 65
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(65)
<223> All pyrimidines are 2'F; feature N at positions
      16-49 is SEQ ID NO: 115 and A, 2'-FC, G or 2'-FU.

<400> 10
gggaggacga ugcggnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnc agacgacucg 60
cccga   65

<210> 11
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<400> 11
cgcaggatcc taatacgact cactata   27

<210> 12
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<400> 12
ggcagaattc tcatctactt agtcgggcga gtcgtctg   38

```
<210> 13
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<400> 13
agcggataac aatttcacac agg                                    23

<210> 14
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<400> 14
gtgctgcaag gcgattaagt tgg                                    23

<210> 15
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<400> 15
actttatgct tccggctcg                                         19

<210> 16
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<400> 16
gccaaatgcc gagagaacg                                         19
```

```
<210>  17
<211>  18
<212>  DNA
<213>  Artificial Sequence

<220>
<223>  Description of Artificial Sequence:  Synthetic
       Sequence

<400>  17
ggggacaagc ggacttag                                                    18

<210>  18
<211>  18
<212>  DNA
<213>  Artificial Sequence

<220>
<223>  Description of Artificial Sequence:  Synthetic
       Sequence

<400>  18
gggagtacag ctatacag                                                    18

<210>  19
<211>  15
<212>  RNA
<213>  Artificial Sequence

<220>
<223>  Description of Artificial Sequence:  Synthetic
       Sequence

<220>
<221>  modified_base
<222>  (1)..(15)
<223>  Bases at positions 5-15 are 2'-OMe.

<400>  19
ccgcaugcuc cuccc                                                       15

<210>  20
<211>  14
<212>  RNA
<213>  Artificial Sequence

<220>
<223>  Description of Artificial Sequence:  Synthetic
```

        Sequence

 <220>
 <221> modified_base
 <222> (1)..(12)
 <223> Bases at positions 1-12 are 2'-OMe.

 <400> 20
 ucgggcgagu cgcg   14

 <210> 21
 <211> 61
 <212> RNA
 <213> Artificial Sequence

 <220>
 <223> Description of Artificial Sequence: Synthetic
        Sequence

 <220>
 <221> modified_base
 <222> (1)..(61)
 <223> All pyrimidines are 2'F.

 <400> 21
 gggaggacga ugcgguccuc aaugaucuuu ccuguuuaug cuccccagac gacucgcccg 60
 a   61

 <210> 22
 <211> 71
 <212> RNA
 <213> Artificial Sequence

 <220>
 <223> Description of Artificial Sequence: Synthetic
        Sequence.

 <220>
 <221> modified_base
 <222> (1)..(71)
 <223> All pyrimidines are 2'F.

 <400> 22
 gggaggacga ugcggaagua acguuuaagu aaaauucguu cucucgguau uuggccagac 60
 gacucgcccg a   71

 <210> 23
 <211> 71

                                    8

<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
     Sequence

<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.

<400> 23
gggaggacga ugcggaagua acguugaagu aaaauucguu cucucggcau uuggccagac 60
gacucgcccg a   71

<210> 24
<211> 71
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
     Sequence

<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.

<400> 24
gggaggacga ugcgguccua accaucacaa ucucaauucu uauauuuucc cgccccagac 60
gacucgcccg a   71

<210> 25
<211> 69
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
     Sequence

<220>
<221> modified_base
<222> (1)..(69)
<223> All pyrimidines are 2'F.

```
<400> 25
gggaggacga ugcggaaacc aaaagaccac aucuccauac ucacgcucug ccccagacga 60
cucgcccga                                                          69
```

```
<210> 26
<211> 71
<212> RNA
<213> Artificial Sequence
```

```
<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence
```

```
<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.
```

```
<400> 26
gggaggacga ugcggauaga ucgguccgau aagucuuuca ucuuuaccug gcccccagac 60
gacucgcccg a                                                        71
```

```
<210> 27
<211> 71
<212> RNA
<213> Artificial Sequence
```

```
<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence
```

```
<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.
```

```
<400> 27
gggaggacga ugcggaagua acguugaagu aaaauucguu cucucgguau uuggccagac 60
gacucgcccg a                                                        71
```

```
<210> 28
<211> 71
<212> RNA
<213> Artificial Sequence
```

```
<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence
```

<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.

<400> 28
gggaggacga ugcggacgau ccuuuccuua acauuucauc auuucuccug ugccccagac 60
gacucgcccg g   71

<210> 29
<211> 71
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.

<400> 29
gggaggacga ugcgguccau caacaaucuu aucauuaugu uuuuccuucc cgccccagac 60
gacucgcccg a   71

<210> 30
<211> 71
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.

<400> 30
gggaggacga ugcgguccuc ugagccgauc uucuucacua cuucuuuuuc ugccccagac 60
gacucgcccg a   71

<210> 31
<211> 71

```
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.

<400> 31
gggaggacga ugcgguuccu caauucuucc aucuucauaa uguuucccuu ugccccagac 60
gacucgcccg a                                                     71

<210> 32
<211> 71
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.

<400> 32
gggaggacga ugcggucuac ccuuuagcag uauuuguuuc caucguuguu ugccccagac 60
gacucgcccg g                                                     71

<210> 33
<211> 71
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.
```

<400> 33
gggaggacga ugcggucuca acgaagaaca ucguuggaua cuguuugucc cgccccagac 60
gacucgcccg a   71

<210> 34
<211> 71
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.

<400> 34
gggaggacga ugcgguucag uuuccuucag uuuucguuuc uaauucuugu gucccagac 60
gacucgcccg a   71

<210> 35
<211> 61
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(61)
<223> All pyrimidines are 2'F.

<400> 35
gggaggacga ugcggagcgg auuaauuagu cugacuucuu gucccagac gacucgcccg 60
a   61

<210> 36
<211> 71
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.

<400> 36
gggaggacga ugcggagaca ucuuugcuc gauuagucau guuccuuacc ugccccagac 60
gacucgcccg a   71

<210> 37
<211> 69
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(69)
<223> All pyrimidines are 2'F.

<400> 37
gggaggacga ugcgguccuc uagcaagcag cuucucaucu uauuuuuccg ccccagacga 60
cucgcccga   69

<210> 38
<211> 71
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.

<400> 38
gggaggacga ugcggugcac agugauggau gacauuguau aacgguaugc gucccagac 60
gacucgcccg a   71

<210> 39
<211> 70

14

```
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:   Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidines are 2'F.

<400> 39
gggaggacga ugcggaccua ucuuucuucc aagucauagu uuuacuuccc gccccagacg 60
acucgcccga                                                      70

<210> 40
<211> 71
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:   Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.

<400> 40
gggaggacga ugcggaugag accuaaucau cgauccgcua ucuaaaaccu caccccagac 60
gacucgcccg a                                                    71

<210> 41
<211> 71
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:   Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.
```

15

```
<400> 41
gggaggacga ugcgguccuc agacaaaucu uucuugaauc uuuccuuaac ugccccagac 60
gacucgcccg a                                                      71

<210> 42
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:   Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidines are 2'F.

<400> 42
gggaggacga ugcggaccga uucuccaacu ugacauuuau uccucuuucu gccccagacg 60
acucgcccga                                                        70

<210> 43
<211> 71
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:   Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.

<400> 43
gggaggacga ugcgguccuc ugagccaauc uucuucgcua cuucuuuuc ugccccagac 60
gacucgcccg a                                                      71

<210> 44
<211> 71
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:   Synthetic
      Sequence
```

16

```
<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.

<400> 44
gggaggacga ugcggauucu uucuccaacg cuuuucacua ccuacauuuc ugccccagac 60
gacucgcccg a                                                        71

<210> 45
<211> 71
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:    Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.

<400> 45
gggaggacga ugcggauccu auccucugaa uaucauuaaa ucaucuucuc cgccccagac 60
gacucgcccg a                                                        71

<210> 46
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:    Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidines are 2'F.

<400> 46
gggaggacga ugcgguucaa ucaucuucac ucucauuucc uuuuuccuac ucccagacg 60
acucgcccga                                                         70

<210> 47
<211> 71
```

17

```
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.

<400> 47
gggaggacga ugcggcgaua gaaucuaguc guucuagaug aucugguacg ugccccagac 60
gacucgcccg a                                                       71

<210> 48
<211> 71
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.

<400> 48
gggaggacga ugcgguagua auccuugucu uccauuucuc uuuacccuuu ugccccagac 60
gacucgcccg a                                                       71

<210> 49
<211> 61
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(61)
<223> All pyrimidines are 2'F.
```

<400> 49
gggaggacga ugcggcccau uaguccucau uagucccug ugccccagac gacucgcccg 60
a   61

<210> 50
<211> 71
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.

<400> 50
gggaggacga ugcggcaucu uauccuccau caguuacucu ucguuauucc cgccccagac 60
gacucgcccg a   71

<210> 51
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidines are 2'F.

<400> 51
gggaggacga ugcgguccaa auccucuucc cauguuagca uucagccuug ucccagacg 60
acucgcccga   70

<210> 52
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

19

```
<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidines are 2'F.


<400> 52
gggaggacga ugcgguuccg acaauuuccu ccaccauuag auuucuugcu gccccagacg 60
acucgcccga                                                      70


<210> 53
<211> 71
<212> RNA
<213> Artificial Sequence


<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence


<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.


<400> 53
gggaggacga ugcggucuug auccuccuuu gugucuuucu uugucuuccc ugccccagac 60
gacucgcccg a                                                    71


<210> 54
<211> 70
<212> RNA
<213> Artificial Sequence


<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence


<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidines are 2'F.


<400> 54
gggaggacga ugcggaagua acguugaagu aaaauucguu cucucgguau uggccagacg 60
acucgcccga                                                      70


<210> 55
<211> 70
```

```
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:    Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidines are 2'F.

<400> 55
gggaggacga ugcgguccga ucaguuccuu cgauuaaucu ucuuuccugc cccccagacg 60
acucgcccga                                                      70

<210> 56
<211> 71
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:    Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.

<400> 56
gggaggacga ugcggaaucc uucucccuga ugaauaugac cuuuuucuug cuccccagac 60
gacucgcccg a                                                    71

<210> 57
<211> 71
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:    Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.
```

```
<400> 57
gggaggacga ugcggaugau cuuuaauguc ugguuugagg ucaaugcggg ugccccagac 60
gacucgcccg a                                                      71

<210> 58
<211> 71
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.

<400> 58
gggaggacga ugcggagaug guacuccauc uccuuuaugu gcccaucgcu guccccagac 60
gacucgcccg a                                                      71

<210> 59
<211> 61
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(61)
<223> All pyrimidines are 2'F.

<400> 59
gggaggacga ugcgguccuc gauucuaauu uacuccuuuu uccccagac gacucgcccg 60
a                                                                 61

<210> 60
<211> 71
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence
```

22

<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.

<400> 60
gggaggacga ugcggucuac ccuuuagcag uauuuguuuc caucguuguu ugccccagac 60
gacucgcccg a   71

<210> 61
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidines are 2'F.

<400> 61
gggaggacga ugcggcacaa uauucuccuc uacuuccacg uauuuuccug uccccagacg 60
acucgcccga   70

<210> 62
<211> 71
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.

<400> 62
gggaggacga ugcgguccuc aaccuuagac uuucauuucu ucaguucuuc ugccccagac 60
gacucgcccg a   71

<210> 63
<211> 71

```
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:   Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.

<400> 63
gggaggacga ugcgguagug gucugucaaa ggaauagcua guaguguuug gucccagac 60
gacucgcccg a                                                    71

<210> 64
<211> 71
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:   Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.

<400> 64
gggaggacga ugcggcaucu uccuuagcau accaguuuau uccuuucccu gucccagac 60
gacucgcccg a                                                    71

<210> 65
<211> 71
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:   Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.
```

<400> 65
gggaggacga ugcggagcga caguauaguu aguacucuag cucuagugcu gucccagac 60
gacucgcccg a   71

<210> 66
<211> 71
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
     Sequence

<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.

<400> 66
gggaggacga ugcggaccuc ucaugaucag caucucgcgu aaucacgguu caccccagac 60
gacucgcccg a   71

<210> 67
<211> 71
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
     Sequence

<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.

<400> 67
gggaggacga ugcgguccgu acuccauuuc cuauuugauu ccuuuccuc ugccccagac 60
gacucgcccg a   71

<210> 68
<211> 71
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
     Sequence

```
<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.


<400> 68
gggaggacga ugcggaaccc acgaccuuac cuuaaucaug uauuucucuc ugccccagac 60
gacucgcccg a                                                        71


<210> 69
<211> 65
<212> RNA
<213> Artificial Sequence


<220>
<223> Description of Artificial Sequence:   Synthetic
      Sequence


<220>
<221> modified_base
<222> (1)..(65)
<223> All pyrimidines are 2'F.


<400> 69
gggaggacga ugcggagaua augagugacg gugauuauag augcugcccc agacgacucg 60
cccga                                                               65


<210> 70
<211> 71
<212> RNA
<213> Artificial Sequence


<220>
<223> Description of Artificial Sequence:   Synthetic
      Sequence


<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.


<400> 70
gggaggacga ugcgguuccu caauucuucc aucuucauaa uguuucccuu ugccccagac 60
gacucgcccg a                                                        71


<210> 71
<211> 60
```

<210> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(60)
<223> All pyrimidines are 2'F.

<400> 71
gggaggacga ugcgguuccu uccaacguua ucuacuuucu gccccagacg acucgcccga 60

<210> 72
<211> 71
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.

<400> 72
gggaggacga ugcggaagua acguuguagu aaaauucguu cucucggcau uuggccagac 60
gacucgcccg a   71

<210> 73
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidines are 2'F.

<400> 73

gggaggacga ugcggaagua acguuauagu aaaauucguu cucucgguau uggccagacg 60
acucgcccga   70

<210> 74
<211> 71
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.

<400> 74
gggaggacgg ugcggaagua acguuguagu aaaauucguu cucucggcgu uuggccagac 60
gacucgcccg a   71

<210> 75
<211> 71
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.

<400> 75
gggaggacga ugcggaagua acguuguagu aaaauucguu cucucggcgu uuggucagac 60
gacucgcccg a   71

<210> 76
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

```
<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidines are 2'F.


<400> 76
gggagacgau gcggaaguaa cguuguagua aaauucguuc ucucggcauu uggccagacg 60
acucgcccga                                                        70


<210> 77
<211> 71
<212> RNA
<213> Artificial Sequence


<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence


<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.


<400> 77
gggaggacga ugcggaagua acguugaagu aaaauucguu cucucugcgu uuggucagac 60
gacucgcccg a                                                      71


<210> 78
<211> 70
<212> RNA
<213> Artificial Sequence


<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence


<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidines are 2'F.


<400> 78
gggaggacga ugcggaagua acguugaagu aaaauucguu cuccugguau uggccagacg 60
acucgcccga                                                        70


<210> 79
<211> 71
<212> RNA
```

<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.

<400> 79
gggaggacga ugcggaagua acguugaagu aaaauucguu cucucggcau uuggccagac 60
gacucgcccg a   71

<210> 80
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidines are 2'F.

<400> 80
gggaggacga ugcggaagua acguuguagu aaaauucguu cucuuggcau uugccagacg 60
acucgcccga   70

<210> 81
<211> 71
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.

<400> 81

gggaggacga ugcggaagua acguuaaagu aaaauucguu cucucggcgu uuggccagac 60
gacucgcccg a   71

<210> 82
<211> 71
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.

<400> 82
gggaggacga ugcggaagua acguugaagu aaaauucguu cucucggcgu uuggccagac 60
gacucgcccg a   71

<210> 83
<211> 71
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.

<400> 83
gggaggacga ugcggaagua acguuguagu aaaauucguu cucucgguau uuggccagac 60
gacucgcccg a   71

<210> 84
<211> 70
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

```
<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidines are 2'F.


<400> 84
gggaggacga ugcggaagua acguuguagu aaaauucguu cucuuggucu uggccagacg 60
acucgcccga                                                      70


<210> 85
<211> 70
<212> RNA
<213> Artificial Sequence


<220>
<223> Description of Artificial Sequence:  Synthetic
     Sequence


<220>
<221> modified_base
<222> (1)..(70)
<223> All pyrimidines are 2'F.


<400> 85
ggaggacgau gcgaaguaac guuguaguaa aauucguucu cucgggcauu uggcagacga 60
cuucgcccga                                                      70


<210> 86
<211> 71
<212> RNA
<213> Artificial Sequence


<220>
<223> Description of Artificial Sequence:  Synthetic
     Sequence


<220>
<221> modified_base
<222> (1)..(71)
<223> All pyrimidines are 2'F.


<400> 86
gggaggacga ugcggcguug uuuagucgua uguauauacu aaguccgcuu gucccagac 60
gacucgcccg a                                                    71


<210> 87
<211> 70
<212> RNA
```

\<213\> Artificial Sequence

\<220\>
\<223\> Description of Artificial Sequence: Synthetic
      Sequence

\<220\>
\<221\> modified_base
\<222\> (1)..(70)
\<223\> All pyrimidines are 2'F.

\<400\> 87
gggaggacga ugcgguucag gagguuauua cagagucugu auagcuguac uccccagacg 60
acucgcccga   70

\<210\> 88
\<211\> 70
\<212\> RNA
\<213\> Artificial Sequence

\<220\>
\<223\> Description of Artificial Sequence: Synthetic
      Sequence

\<220\>
\<221\> modified_base
\<222\> (1)..(70)
\<223\> All pyrimidines are 2'F.

\<400\> 88
gggaggacga ugcgguucag gaggguauua cagagucugu auagcuguac uccccagacg 60
acucgcccga   70

\<210\> 89
\<211\> 57
\<212\> RNA
\<213\> Artificial Sequence

\<220\>
\<223\> Description of Artificial Sequence: Synthetic
      Sequence

\<220\>
\<221\> modified_base
\<222\> (1)..(57)
\<223\> All pyrimidines are 2'F.

\<400\> 89

gguucaggag guuauuacag agucuguaua gcuguacucc ccagacgacu cgcccga   57

<210> 90
<211> 56
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
     Sequence

<220>
<221> modified_base
<222> (1)..(56)
<223> All pyrimidines are 2'F.

<400> 90
gggaggacga ugcgguucag gagguuauua cagagucugu auagcguac uccca   56

<210> 91
<211> 43
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
     Sequence

<220>
<221> modified_base
<222> (1)..(43)
<223> All pyrimidines are 2'F.

<400> 91
gguucaggag guuauuacag agucuguaua gcuguacucc cca   43

<210> 92
<211> 36
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
     Sequence

<220>
<221> modified_base
<222> (1)..(36)

34

<223> All pyrimidines are 2'F.

<400> 92
ggagguuauu acagagucug uauagcugua. cucccc   36

<210> 93
<211> 34
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
       Sequence

<220>
<221> modified_base
<222> (1)..(34)
<223> All pyrimidines are 2'F.

<400> 93
ggagguuauu acagagucug uauagcugua cucc   34

<210> 94
<211> 30
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
       Sequence

<220>
<221> modified_base
<222> (1)..(30)
<223> All pyrimidines are 2'F.

<400> 94
ggagguuauu acagagucug uauagcugua   30

<210> 95
<211> 26
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
       Sequence

```
<220>
<221> modified_base
<222> (1)..(26)
<223> All pyrimidines are 2'F.

<400> 95
ggagguuauu acagagucug uauagc                                    26

<210> 96
<211> 30
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(30)
<223> All pyrimidines are 2'F.

<400> 96
ggagguuauu acagagucug uauagccucc                                30

<210> 97
<211> 30
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(30)
<223> All pyrimidines are 2'F.

<400> 97
ggagguuauu agagucuaua gcuguacucc                                30

<210> 98
<211> 26
<212> RNA
<213> Artificial Sequence

<220>
```

<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(26)
<223> All pyrimidines are 2'F.

<400> 98
ggagguuauu agagucuaua gccucc   26

<210> 99
<211> 33
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(33)
<223> All pyrimidines are 2'F.

<400> 99
ggagguuauu acagagucug uauagcugua cuc   33

<210> 100
<211> 32
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(32)
<223> All pyrimidines are 2'F.

<400> 100
ggagguuauu acagagucug uauagcugua cu   32

<210> 101
<211> 31
<212> RNA

```
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(31)
<223> All pyrimidines are 2'F.

<400> 101
ggagguuauu acagagucug uauagcugua c                    31

<210> 102
<211> 33
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(33)
<223> All pyrimidines are 2'F.

<400> 102
ggagguuauu acagagucug uauagcguac ucc                  33

<210> 103
<211> 33
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(33)
<223> All pyrimidines are 2'F.

<400> 103
ggagguuauu acagagucug uauagcuguc ucc                  33
```

```
<210> 104
<211> 32
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(32)
<223> All pyrimidines are 2'F.

<400> 104
ggagguuauu acagagucug uauagcuacu cc                              32

<210> 105
<211> 33
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(33)
<223> All pyrimidines are 2'F.

<400> 105
ggagguuaua cagagucugu auagcuguac ucc                             33

<210> 106
<211> 33
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(33)
<223> All pyrimidines are 2'F.
```

<400> 106
ggagguuauu acagaucugu auagcuguac ucc   33

<210> 107
<211> 32
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(32)
<223> All pyrimidines are 2'F.

<400> 107
ggagguuauu acaaguugua uagcuguacu cc   32

<210> 108
<211> 33
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(33)
<223> All pyrimidines are 2'F.

<400> 108
ggagguuauu acagaguugu auagcuguac ucc   33

<210> 109
<211> 32
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base

```
<222> (1)..(32)
<223> All pyrimidines are 2'F.

<400> 109
gggguuauua cagagucugu auagcuguac cc                              32

<210> 110
<211> 32
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:   Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(32)
<223> All pyrimidines are 2'F.

<400> 110
ggagguuauu acgagucugu auagcguacu cc                              32

<210> 111
<211> 33
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:   Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(33)
<223> All pyrimidines are 2'F.

<400> 111
ggagauauua cagagucugu auagcuguac ucc                             33

<210> 112
<211> 30
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:   Synthetic
      Sequence
```

```
<220>
<221> modified_base
<222> (1)..(30)
<223> All pyrimidines are 2'F.

<400> 112
ggggguuauuc agagucugua uagcugaccc                                    30

<210> 113
<211> 30
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(30)
<223> All pyrimidines are 2'F.

<400> 113
ggggguuauua agagucugua uagcuuaccc                                    30

<210> 114
<211> 36
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(36)
<223> All pyrimidines are 2'F.

<400> 114
ggagguuauu acagagucug uauagcugua cuccc                               36

<210> 115
<211> 34
<212> RNA
<213> Artificial Sequence
```

42

```
<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(34)
<223> All pyrimidines are 2'F.

<400> 115
ggagguuauu acagagucug uauagcugua cucc                          34

<210> 116
<211> 37
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(37)
<223> All pyrimidines are 2'F.

<400> 116
ggagguuauu acagagucug uauagcugua cuccca                        37

<210> 117
<211> 30
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(30)
<223> All pyrimidines are 2'F.

<400> 117
ggagguuauu acagagucug uauagcugua                              30

<210> 118
<211> 33
```

```
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(33)
<223> All pyrimidines are 2'F.

<400> 118
gagguuauua cagagucugu auagcuguac ucc                          33

<210> 119
<211> 32
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(32)
<223> All pyrimidines are 2'F.

<400> 119
agguuauuac agagucugua uagcuguacu cc                           32

<210> 120
<211> 31
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(31)
<223> All pyrimidines are 2'F.

<400> 120
gguuauuaca gagucuguau agcuguacuc c                            31
```

```
<210> 121
<211> 34
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:   Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(34)
<223> All pyrimidines are 2'F.

<400> 121
ggagguuauu acagagucug uauagcugua cucc                              34

<210> 122
<211> 60
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:   Synthetic
      Sequence

<400> 122
Ala Leu Asp Thr Asn Tyr Cys Phe Ser Ser Thr Glu Lys Asn Cys Cys
 1               5                  10                  15

Val Arg Gln Leu Tyr Ile Asp Phe Arg Lys Asp Leu Gly Trp Lys Trp
            20                  25                  30

Ile His Glu Pro Lys Gly Tyr His Ala Asn Phe Cys Leu Gly Pro Cys
            35                  40                  45

Pro Tyr Ile Trp Ser Leu Asp Thr Gln Tyr Ser Lys
        50                  55                  60


<210> 123
<211> 60
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:   Synthetic
```

Sequence

<400> 123
Ala Leu Asp Ala Ala Tyr Cys Phe Arg Asn Val Gln Asp Asn Cys Cys
1   5   10   15

Leu Arg Pro Leu Tyr Ile Asp Phe Lys Arg Asp Leu Gly Trp Lys Trp
            20   25   30

Ile His Glu Pro Lys Gly Tyr Asn Ala Asn Phe Cys Ala Gly Ala Cys
        35   40   45

Pro Tyr Leu Trp Ser Ser Asp Thr Gln His Ser Arg
    50   55   60

<210> 124
<211> 60
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<400> 124
Ala Leu Asp Thr Asn Tyr Cys Phe Arg Asn Leu Glu Glu Asn Cys Cys
1   5   10   15

Val Arg Pro Leu Tyr Ile Asp Phe Arg Gln Asp Leu Gly Trp Lys Trp
            20   25   30

Val His Glu Pro Lys Gly Tyr Tyr Ala Asn Phe Cys Ser Gly Pro Cys
        35   40   45

Pro Tyr Leu Arg Ser Ala Asp Thr Thr His Ser Thr
    50   55   60

<210> 125
<211> 52
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

46

<400> 125
Val Leu Ala Leu Tyr Asn Gln His Asn Pro Gly Ala Ser Ala Ala Pro
1   5   10   15

Cys Cys Val Pro Gln Ala Leu Glu Pro Leu Pro Ile Val Tyr Tyr Val
                20   25   30

Gly Arg Lys Pro Lys Val Glu Gln Leu Ser Asn Met Ile Val Arg Ser
            35   40   45

Cys Lys Cys Ser
        50

<210> 126
<211> 52
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
       Sequence

<400> 126
Val Leu Ser Leu Tyr Asn Thr Ile Asn Pro Glu Ala Ser Ala Ser Pro
1   5   10   15

Cys Cys Val Ser Gln Asp Leu Glu Pro Leu Thr Ile Leu Tyr Tyr Ile
                20   25   30

Gly Lys Thr Pro Lys Ile Glu Gln Leu Ser Asn Met Ile Val Lys Ser
            35   40   45

Cys Lys Cys Ser
        50

<210> 127
<211> 52
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
       Sequence

<400> 127
Val Leu Gly Leu Tyr Asn Thr Leu Asn Pro Glu Ala Ser Ala Ser Pro

47

```
        1              5                  10                 15
   Cys Cys Val Pro Gln Asp Leu Glu Pro Leu Thr Ile Leu Tyr Tyr Val
                    20                  25                  30

   Gly Arg Thr Pro Lys Val Glu Gln Leu Ser Asn Met Val Val Lys Ser
               35                  40                  45

   Cys Lys Cys Ser
           50
```

```
<210> 128
<211> 58
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:   Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(58)
<223> All pyrimidines are 2'F.

<400> 128
ggaaguaacg uuguaguaaa auucguucuc ucggcauuug gccagacgac ucgcccga    58

<210> 129
<211> 57
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:   Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(57)
<223> All pyrimidines are 2'F.

<400> 129
gggaggacga ugcggaagua acguuguagu aaaauucguu cucucggcau uuggcca    57

<210> 130
<211> 44
```

```
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:   Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(44)
<223> All pyrimidines are 2'F.

<400> 130
ggaaguaacg uuguaguaaa auucguucuc ucggcauuug gcca            44

<210> 131
<211> 38
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:   Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(38)
<223> All pyrimidines are 2'F.

<400> 131
gggaggaugc ggaaguaacg uuguaguaaa auuccuuc                   38

<210> 132
<211> 35
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:   Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(35)
<223> All pyrimidines are 2'F.

<400> 132
gggaggaugc ggaaguaacg uuguaguaaa auucc                      35
```

```
<210> 133
<211> 32
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:   Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(32)
<223> All pyrimidines are 2'F.

<400> 133
gggaggaugc ggaaguaacg uuguaguaaa au                                    32

<210> 134
<211> 33
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:   Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(33)
<223> All pyrimidines are 2'F.

<400> 134
gggaggaugc ggaaguaacg uuguaguucc uuc                                   33

<210> 135
<211> 27
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:   Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(27)
<223> All pyrimidines are 2'F.
```

```
<400> 135
gggaggaugc ggaaguaacg uuguagu                                                    27

<210> 136
<211> 20
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(20)
<223> All pyrimidines are 2'F.

<400> 136
gggaggagua acguuguagu                                                            20

<210> 137
<211> 58
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(58)
<223> All pyrimidines are 2'F.

<400> 137
ggcguuguuu agucguaugu auauacuaag uccgcuuguc cccagacgac ucgcccga      58

<210> 138
<211> 57
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
```

```
<221> modified_base
<222> (1)..(57)
<223> All pyrimidines are 2'F.

<400> 138
gggaggacga ugcggcguug uuuagucgua uguauauacu aaguccgcuu gucccca        57

<210> 139
<211> 44
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:   Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(44)
<223> All pyrimidines are 2'F.

<400> 139
ggcguuguuu agucguaugu auauacuaag uccgcuuguc ccca                      44

<210> 140
<211> 44
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:   Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(44)
<223> All pyrimidines are 2'F.

<400> 140
ggggagcggc guuguuuagu cguauguaua uacuaagucc gcuu                      44

<210> 141
<211> 29
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:   Synthetic
```

Sequence

```
<220>
<221> modified_base
<222> (1)..(29)
<223> All pyrimidines are 2'F.


<400> 141
ggggagcggc guuguugaaa aguccgcuu                        29

<210> 142
<211> 38
<212> RNA
<213> Artificial Sequence


<220>
<223> Description of Artificial Sequence:  Synthetic
     Sequence

<220>
<221> modified_base
<222> (1)..(38)
<223> All pyrimidines are 2'F.


<400> 142
ggggagcggc guuguuucgu auguauauaa guccgcuu              38

<210> 143
<211> 33
<212> RNA
<213> Artificial Sequence


<220>
<223> Description of Artificial Sequence:  Synthetic
     Sequence

<220>
<221> modified_base
<222> (1)..(33)
<223> All pyrimidines are 2'F.


<400> 143
ggggagcggc guuguuuaug uauaaguccg cuu                   33

<210> 144
<211> 42
<212> RNA
<213> Artificial Sequence
```

```
<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(42)
<223> All pyrimidines are 2'F.

<400> 144
ggggagcggc guuguuuagu cguauguaua uacuaagucc gc                    42

<210> 145
<211> 38
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(38)
<223> All pyrimidines are 2'F.

<400> 145
ggggagcggc guuguuuagu cguauguaua uacuaagu                         38

<210> 146
<211> 65
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(65)
<223> All pyrimidines are 2'F.

<400> 146
gggaggacga ugcgggguga uuauuacaga guauguauag cuguacccc agacgacucg 60
cccga                                                            65
```

```
<210> 147
<211> 64
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(64)
<223> All pyrimidines are 2'F.

<400> 147
gggaggacga ugcggaggcg uuauuagaga gucuguauag cucuagcccc agacgacucg 60
ccga                                                                 64

<210> 148
<211> 65
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(65)
<223> All pyrimidines are 2'F.

<400> 148
gggaggacga ugcggggagg guauuacaga guauguauag cuguacuccc agacgacucg 60
cccga                                                                65

<210> 149
<211> 65
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(65)
```

<223> All pyrimidines are 2'F.

<400> 149
gggaggacga ugcggggagg uuauuauaga gucuguauag cuauacccc agacgacucg 60
cccga   65

<210> 150
<211> 65
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(65)
<223> All pyrimidines are 2'F.

<400> 150
gggaggacga ugcgggaggg uuauuauaga gucugcauag cuauacccc agacgacucg 60
cccga   65

<210> 151
<211> 65
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(65)
<223> All pyrimidines are 2'F.

<400> 151
gggaggacga ugcggugaga guauuacgga guauguauag ccguacccc agacgacucg 60
cccga   65

<210> 152
<211> 65
<212> RNA
<213> Artificial Sequence

<220>

56

```
<223> Description of Artificial Sequence:  Synthetic
      Sequence


<220>
<221> modified_base
<222> (1)..(65)
<223> All pyrimidines are 2'F.


<400> 152
gggaggacga ugcgggggca uuauuucaga gucuguauag cuguagcccc agacgacucg 60
cccga                                                        65


<210> 153
<211> 65
<212> RNA
<213> Artificial Sequence


<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence


<220>
<221> modified_base
<222> (1)..(65)
<223> All pyrimidines are 2'F.


<400> 153
gggaggacga ugcgggcgga uuaucacaga guauguauag cugugccgcc agacgacucg 60
cccga                                                        65


<210> 154
<211> 65
<212> RNA
<213> Artificial Sequence


<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence


<220>
<221> modified_base
<222> (1)..(65)
<223> All pyrimidines are 2'F.


<400> 154
gggaggacga ugcggguguga auauuagaga gucuguauag cucuacccc agacgacucg 60
cccga                                                        65
```

<210> 155
<211> 65
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(65)
<223> All pyrimidines are 2'F.

<400> 155
gggaggacga ugcggcggga uuauuacuga gucuguauag caguaccccc agacgacucg 60
cccga   65

<210> 156
<211> 65
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(65)
<223> All pyrimidines are 2'F.

<400> 156
gggaggacga ugcgggugga auauuacgga gucuguauag ccguacuccc agacgacucg 60
cccga   65

<210> 157
<211> 65
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(65)

<223> All pyrimidines are 2'F.

<400> 157
gggaggacga ugcgggggga cuauuaguga gucuguauag cacuaccccc agacgacucg 60
cccga   65

<210> 158
<211> 65
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(65)
<223> All pyrimidines are 2'F.

<400> 158
gggaggacga ugcgggugga uuauuacagc gucuguauau cuguacccccc agacgacucg 60
cccga   65

<210> 159
<211> 65
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(65)
<223> All pyrimidines are 2'F.

<400> 159
gggaggacga ugcgggcagg uuauuacaga gucuguauag cuguacugcc agacgacucg 60
cccga   65

<210> 160
<211> 65
<212> RNA
<213> Artificial Sequence

<220>

<223> D scription of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(65)
<223> All pyrimidines are 2'F.

<400> 160
gggaggacga ugcgggguag auaucacuga gucuguauag caguguccccc agacgacucg 60
cccga   65

<210> 161
<211> 65
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(65)
<223> All pyrimidines are 2'F.

<400> 161
gggaggacga ugcggaggga uuauuacaga gucuguauag cuguaccccc agacgacucg 60
cccga   65

<210> 162
<211> 65
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(65)
<223> All pyrimidines are 2'F.

<400> 162
gggaggacga ugcgggugga uuauuacaga gucuguauag cuguaccccc agacgacucg 60
cccga   65

```
<210> 163
<211> 65
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(65)
<223> All pyrimidines are 2'F.

<400> 163
gggaggacga ugcgggggcg uuauuacaga gucuguauag cuguagcccc agacgacucg 60
cccga                                                              65

<210> 164
<211> 65
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(65)
<223> All pyrimidines are 2'F.

<400> 164
gggaggacga ugcggggugg uuauuacaca guauguauag guguaccccc agacgacucg 60
cccga                                                              65

<210> 165
<211> 65
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(65)
```

<223> All pyrimidines are 2'F.

<400> 165
gggaggacga ugcggaggga auauuacaga guauguauag cguuaccccc agacgacucg 60
cccga   65

<210> 166
<211> 65
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(65)
<223> All pyrimidines are 2'F.

<400> 166
gggaggacga ugcggggagu uuauuacagc gucuguauau cguuagcccc agacgacucg 60
cccga   65

<210> 167
<211> 65
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(65)
<223> All pyrimidines are 2'F.

<400> 167
gggaggacga ugcggugagg uuauuacaga gucuguauag cguuacuccc agacgacucg 60
cccga   65

<210> 168
<211> 65
<212> RNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic
     Sequence

<220>
<221> modified_base
<222> (1)..(65)
<223> All pyrimidines are 2'F.

<400> 168
gggaggacga ugcggggugg uuauuagaga gucuguauag cucuacgccc agacgacucg 60
cccga   65

<210> 169
<211> 65
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
     Sequence

<220>
<221> modified_base
<222> (1)..(65)
<223> All pyrimidines are 2'F.

<400> 169
gggaggacga ugcggggggga guauuaaaga gucuguauag cuuuaccccc agacgacucg 60
cccga   65

<210> 170
<211> 65
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
     Sequence

<220>
<221> modified_base
<222> (1)..(65)
<223> All pyrimidines are 2'F.

<400> 170
gggaggacga ugcggggagg auauuauaga gucuguauag cuauaccccc agacgacucg 60
cccga   65

<210> 171
<211> 34
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(34)
<223> All pyrimidines are 2'F; a's and g's at positions
      1-5, 8, 11, 13-16, 20, 22, 24-25, 28 and 30 are
      2'-OCH3; linkage at positions 34 and 35 is 3'-3'.

<400> 171
ggagguuauu acagagucug uauagcugua cucc   34

<210> 172
<211> 34
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(34)
<223> All pyrimidines are 2'F; a's and g's at positions
      11, 13-16, 20, 22, 24-25, 28 and 30 are 2'OCH3;
      linkage at positions 34.and 35 is 3'-3'.

<400> 172
ggagguuauu acagagucug uauagcugua cucc   34

<210> 173
<211> 34
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>

```
<221> modified_base
<222> (1)..(34)
<223> All pyrimidines are 2'F; a's and g's at positions
      1-4 are 2'OCH3; linkage at positions 34 and 35 is
      3'-3'.

<400> 173
ggagguuauu acagagucug uauagcugua cucc                              34

<210> 174
<211> 34
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:   Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(34)
<223> All pyrimidines are 2'F; a's and g's at positions
      5, 8, and 11 are 2'OCH3; linkage at positions 34
      and 35 is 3'-3'.

<400> 174
ggagguuauu acagagucug uauagcugua cucc                              34

<210> 175
<211> 34
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:   Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(34)
<223> All pyrimidines are 2'F; a' and g's at positions
      13-16 are 2'-OCH3; linkage at positions 34 and 35
      is 3'-3'.

<400> 175
ggagguuauu acagagucug uauagcugua cucc                              34

<210> 176
```

```
<211> 34
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(34)
<223> All pyrimidines are 2'F; a's and g's at positions
      20, 22 and 24 are 2'-OCH3; linkage at positions 34
      and 35 is 3'-3'.

<400> 176
ggagguuauu acagagucug uauagcugua cucc                                  34

<210> 177
<211> 34
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(34)
<223> All pyrimidines are 2'F; a's and g's at positions
      25, 28 and 30 are 2'-OCH3; linkage at positions 34
      and 35 is 3'-3'.

<400> 177
ggagguuauu acagagucug uauagcugua cucc                                  34

<210> 178
<211> 34
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
```

```
<222> (1)..(34)
<223> All pyrimidines are 2'F; g at position 20 is
      2'-OCH3; linkage at positions 34 and 35 is 3'-3'.


<400> 178
ggagguuauu acagagucug uauagcugua cucc                              34


<210> 179
<211> 34
<212> RNA
<213> Artificial Sequence


<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence


<220>
<221> modified_base
<222> (1)..(34)
<223> All pyrimidines are 2'F; a at position 22 is
      2'-OCH3; linkage at positions 34 and 35 is 3'-3'.


<400> 179
ggagguuauu acagagucug uauagcugua cucc                              34


<210> 180
<211> 34
<212> RNA
<213> Artificial Sequence


<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence


<220>
<221> modified_base
<222> (1)..(34)
<223> All pyrimidines are 2'F; a at position 24 is
      2'-OCH3; linkage at positions 34 and 35 is 3'-3'.


<400> 180
ggagguuauu acagagucug uauagcugua cucc                              34


<210> 181
<211> 34
<212> RNA
<213> Artificial Sequence
```

```
<220>
<223> Description of Artificial Sequence:   Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(34)
<223> All pyrimidines are 2'F; a's and g's at positions
      1-5,  8,  11, 25 and 30 are 2'-OCH3; linkage at
      positions 34 and 35 is 3'-3'.

<400> 181
ggagguuauu acagagucug uauagcugua cucc                              34

<210> 182
<211> 34
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:   Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(34)
<223> All pyrimidines are 2'F; a's and g's at positions
      1-5,  8,  11, 13-16, 24-25, 28 and 30 are 2'-OCH3;
      linkage at positions 34 and 35 is 3'-3'.

<400> 182
ggagguuauu acagagucug uauagcugua cucc                              34

<210> 183
<211> 34
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:   Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(34)
<223> All pyrimidines are 2'F; a's and g's at positions
      1-5,  8,  11, 13-16, 20, 24-25, 28 and 30 are
      2'-OCH3; linkage at positions 34 and 35 is 3'-3'.
```

68

<400> 183
ggagguuauu acagagucug uauagcugua cucc   34

<210> 184
<211> 34
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(34)
<223> All pyrimidines are 2'F; a's and g's at positions
      1-5, 8, 11, 13-16, 25, 28 and 30 are 2'-OCH3;
      linkage at positions 34 and 35 is 3'-3'.

<400> 184
ggagguuauu acagagucug uauagcugua cucc   34

<210> 185
<211> 34
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(34)
<223> All pyrimidines are 2'F; a's and g's at positions
      1-5, 11, 13-16, 25, 28, and 30 are 2'-OCH3;
      linkage at positions 34 and 35 is 3'-3'.

<400> 185
ggagguuauu acagagucug uauagcugua cucc   34

<210> 186
<211> 34
<212> RNA
<213> Artificial Sequence

<220>

```
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(34)
<223> All pyrimidines are 2'F; a's and g's at positions
      1-4, 13-16, 25, 28 and 30 are 2'-OCH3; linkage at
      positions 34 and 35 is 3'-3'.

<400> 186
ggagguuauu acagagucug uauagcugua cucc                        34

<210> 187
<211> 33
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(33)
<223> All pyrimidines are 2'F; a's and g's at positions
      1-5, 10, 12-15 24, 27 and 29 are 2'-OCH3; linkage
      at positions 33 and 34 is 3'-3'.

<400> 187
ggagauauua cagagucugu auagcguac ucc                          33

<210> 188
<211> 32
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(32)
<223> All pyrimidines are 2'F; a's and g's at positions
      1-4, 10, 12-15, 24, 27 and 29 are 2'-OCH3; linkage
      at positions 32 and 33 is 3'-3'.
```

```
<400> 188
gggguuauua cagagucugu auagcuguac cc                                    32

<210> 189
<211> 34
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(34)
<223> All pyrimidines are 2'F; linkage at positions 34
      and 35 is 3'-3'.

<400> 189
ugugaauauu agagagucug uauagcucua cccc                                  34

<210> 190
<211> 34
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(34)
<223> All pyrimidines are 2'F; a's and g's at positions
      2, 4-5, 8, 11, 13-16, 24-25 and 30 are 2'-OCH3;
      linkage at positons 34 and 35 is 3'-3'.

<400> 190
ugugaauauu agagagucug uauagcucua cccc                                  34

<210> 191
<211> 34
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence
```

```
<220>
<221> modified_base
<222> (1)..(34)
<223> All pyrimidines are 2'F; a's and g's at positions
      2, 4-6, 8, 11-16, 20, 24-25, and 30 are 2'-OCH3;
      linkage at positions 34 and 35 is 3'-3'.

<400> 191
ugugaauauu agagagucug uauagcucua cccc                                    34

<210> 192
<211> 34
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:   Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(34)
<223> All pyrimidines are 2'F; a's and g's at positions
      2, 4, 11, 13-16, 25 and 30 are 2'-OCH3; linkage at
      positions 34 and 35 is 3'-3'.

<400> 192
ugugaauauu agagagucug uauagcucua cccc                                    34

<210> 193
<211> 34
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:   Synthetic
      Sequence

<220>
<221> modified_base
<222> (1)..(34)
<223> All pyrimidines are 2'F; a's and g's at positions
      2, 4-6, 11-16, 25 and 30 are 2'-OCH3; linkage at
      positions 34 and 35 is 3'-3'.

<400> 193
ugugaauauu agagagucug uauagcucua cccc                                    34
```

<210> 194
<211> 42
<212> RNA
<213> Artificial Sequence

<220>
<221> modified_base
<222> (1)..(42)
<223> A's and g's at positions 1-3 and 5 are 2'-OMe.

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<400> 194
gggugccuuu ugccuagguu gugauuugua accuucugcc ca   42

<210> 195
<211> 42
<212> RNA
<213> Artificial Sequence

<220>
<221> modified_base
<222> (1)..(42)
<223> A's and g's at positions 12 and 16-18 are 2'OMe.

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<400> 195
gggugccuuu ugccuagguu gugauuugua accuucugcc ca   42

<210> 196
<211> 42
<212> RNA
<213> Artificial Sequence

<220>
<221> modified_base
<222> (1)..(42)
<223> A's and g's at positions 21, 23-24 and 28 are
      2'-OMe.

<220>
<223> Description of Artificial Sequence: Synthetic

73

Sequence

<400> 196
gggugccuuu ugccuagguu gugauuugua accuucugcc ca   42

<210> 197
<211> 42
<212> RNA
<213> Artificial Sequence

<220>
<221> modified_base
<222> (1)..(42)
<223> A's and g's at positions 30-31, 38 and 42 are
      2'-OMe.

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<400> 197
gggugccuuu ugccuagguu gugauuugua accuucugcc ca   42

<210> 198
<211> 42
<212> RNA
<213> Artificial Sequence

<220>
<221> modified_base
<222> (1)..(42)
<223> A's and g's at positions 1, 21, 23-24 and 28 are
      2'-OMe.

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<400> 198
gggugccuuu ugccuagguu gugauuugua accuucugcc ca   42

<210> 199
<211> 42
<212> RNA
<213> Artificial Sequence

<220>
<221> modified_base

```
<222>  (1)..(42)
<223>  A's and g's at positions 2, 21, 23-24 and 28 are
       2'-OMe.

<220>
<223>  Description of Artificial Sequence:  Synthetic
       Sequence

<400>  199
ggguugccuuu ugccuagguu gugauuugua accuucugcc ca                              42

<210>  200
<211>  42
<212>  RNA
<213>  Artificial Sequence

<220>
<221>  modified_base
<222>  (1)..(42)
<223>  A's and g's at positions 3, 21, 23-24 and 28 are
       2'-OMe.

<220>
<223>  Description of Artificial Sequence:  Synthetic
       Sequence

<400>  200
ggguugccuuu ugccuagguu gugauuugua accuucugcc ca                              42

<210>  201
<211>  42
<212>  RNA
<213>  Artificial Sequence

<220>
<221>  modified_base
<222>  (1)..(42)
<223>  A's and g's at positions 5, 21, 23-24 and 28 are
       2'-OMe.

<220>
<223>  Description of Artificial Sequence:  Synthetic
       Sequence

<400>  201
ggguugccuuu ugccuagguu gugauuugua accuucugcc ca                              42

<210>  202
```

```
<211> 42
<212> RNA
<213> Artificial Sequence

<220>
<221> modified_base
<222> (1)..(42)
<223> A's and g's at positions 12, 21, 23-24 and 28 are
      2'-OMe.

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<400> 202
gggugccuuu ugccuagguu gugauuugua accuucugcc ca                    42

<210> 203
<211> 42
<212> RNA
<213> Artificial Sequence

<220>
<221> modified_base
<222> (1)..(42)
<223> A's and g's at positions 16, 21, 23-24 and 28 are
      2'-OMe.

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<400> 203
gggugccuuu ugccuagguu gugauuugua accuucugcc ca                    42

<210> 204
<211> 42
<212> RNA
<213> Artificial Sequence

<220>
<221> modified_base
<222> (1)..(42)
<223> A's and g's at positions 17, 21, 23-24 and 28 are
      2'-OMe.

<220>
<223> Description of Artificial Sequence:  Synthetic
```

Sequence

<400> 204
ggguugccuuu ugccuagguu gugauuugua accuucugcc ca   42

<210> 205
<211> 42
<212> RNA
<213> Artificial Sequence

<220>
<221> modified_base
<222> (1)..(42)
<223> A's and g's at positions 18, 21, 23-24, and 28 are
      2'-OMe.

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<400> 205
ggguugccuuu ugccuagguu gugauuugua accuucugcc ca   42

<210> 206
<211> 42
<212> RNA
<213> Artificial Sequence

<220>
<221> modified_base
<222> (1)..(42)
<223> A's and g's at positions 21, 23-24, 28 and 30 are
      2'-OMe.

<220>
<223> Description of Artificial Sequence: Synthetic
      Sequence

<400> 206
ggguugccuuu ugccuagguu gugauuugua accuucugcc ca   42

<210> 207
<211> 42
<212> RNA
<213> Artificial Sequence

<220>
<221> modified_base

```
<222> (1)..(42)
<223> A's and g's at positions 21, 23-24, 28 and 31 are
      2'-OMe.

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<400> 207
gggugccuuu ugccuagguu gugauuugua accuucugcc ca                           42

<210> 208
<211> 42
<212> RNA
<213> Artificial Sequence

<220>
<221> modified_base
<222> (1)..(42)
<223> A's and g's at positions 21, 23-24, 28 and 38 are
      2'-OMe.

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<400> 208
gggugccuuu ugccuagguu gugauuugua accuucugcc ca                           42

<210> 209
<211> 42
<212> RNA
<213> Artificial Sequence

<220>
<221> modified_base
<222> (1)..(42)
<223> A's and g's at positions 21, 23-24, 28 and 42 are
      2'-OMe.

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<400> 209
gggugccuuu ugccuagguu gugauuugua accuucugcc ca                           42

<210> 210
```

```
<211> 42
<212> RNA
<213> Artificial Sequence

<220>
<221> modified_base
<222> (1)..(42)
<223> A's and g's at positions 1-3, 16-18, 21, 23-24,
      28, 30-31 and 42 are 2'-OMe; linkage at positions
      42 and 43 is 3'-3'.

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<400> 210
gggugccuuu ugccuagguu gugauuugua accuucugcc ca                    42

<210> 211
<211> 42
<212> RNA
<213> Artificial Sequence

<220>
<221> modified_base
<222> (1)..(42)
<223> A's and g's at positions 1-3, 16-18, 21, 23-24,
      28, 30 and 42 are 2'-OMe; linkage at positions 42
      and 43 is 3'-3'.

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<400> 211
gggugccuuu ugccuagguu gugauuugua accuucugcc ca                    42

<210> 212
<211> 41
<212> RNA
<213> Artificial Sequence

<220>
<221> modified_base
<222> (1)..(41)
<223> A's and g's at positions 1-3, 16-18, 21, 23-24, 28
      and 30-31 are 2'-OMe; linkage at positions 41 and
      42 are 3'-3'.
```

```
<220>
<223> Description of Artificial Sequence:   Synthetic
      Sequence

<400> 212
gggugccuuu ugccuagguu gugauuugua accuucugcc c                           41

<210> 213
<211> 37
<212> RNA
<213> Artificial Sequence

<220>
<221> modified_base
<222> (1)..(37)
<223> A's and g's at positions 1-3, 16-18, 21, 23, 25-26
      and 37 are 2'-OMe; linkage at positions 37 and 38
      is 3'-3'.

<220>
<223> Description of Artificial Sequence:   Synthetic
      Sequence

<400> 213
gggugccuuu ugccuagguu guguaaccuu cugccca                                37

<210> 214
<211> 35
<212> RNA
<213> Artificial Sequence

<220>
<221> modified_base
<222> (1)..(35)
<223> A's and g's at positions 1-3, 16-18, 21, 23-24 and
      35 are 2'-OMe; linkage at positions 35 and 36 is
      3'-3'.

<220>
<223> Description of Artificial Sequence:   Synthetic
      Sequence

<400> 214
gggugccuuu ugccuagguu guaaccuucu gccca                                  35

<210> 215
<211> 33
```

```
<212> RNA
<213> Artificial Sequence

<220>
<221> modified_base
<222> (1)..(33)
<223> A's and g's at positions 1-3, 16-18, 21-22, and 33
      are 2'-OMe; linkage at positions 33 and 34 is
      3'-3'.

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<400> 215
gggugccuuu ugccuagguu aaccuucugc cca                                33

<210> 216
<211> 42
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:  Synthetic
      Sequence

<400> 216
gggugccuuu ugccuagguu gugauuugua accuucugcc ca                      42
```

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C07H 21/02, 21/04

US CL :536/22.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/22.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, DIALOG ONESEARCH; search terms: nucleic ligand, aptamer, TGFbeta2, polyethylene glycol, lipophilic, nonimmunogenic, polyalkylene glycol

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,731,424 A (TOOTHMAN et al.) 24 March 1998, claims 1-9; col. 5, line 66-col. 6, line 1.	2-7
Y	US 5,859,228 A (JANJIC et al.) 12 January 1999; see entire document.	2-7

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
		"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 OCTOBER 2000	24 NOV 2000

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	STEPHANIE ZITOMER, PHD
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)*